# Stabilisation of the Biosensor Properties of Protoplasts Used as the Biological Units of the Protoplast Biosensor

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# 1 INTRODUCTION

Since the beginning of the Industrial Revolution 300 years ago, the problems of water pollution have expanded from being regional and later continental, to global. Especially urbanisation and the consequent increase in population, industrial growth, and agricultural intensification can lead to greater levels of freshwater pollution (UNEP, 1991). It has been estimated that 30,000 deaths around the world are caused daily due to contaminated water and poor sanitation (The Green Lane, 2000). In the USA alone more than 4,000 tonnes of persistent toxic metals, nearly 450 tonnes of reproductive toxins and over 1,250 tonnes of carcinogens were released into the waterways (PIRG, 1999). More than 14 million Americans drink water contaminated with pesticides (Hart, 2000).

In order to protect humans and the environment from pollution the World Health Organisation (WHO) has been concerned with water quality for over 45 years. The International Standards for Drinking-Water were publicised in 1958 (WHO, 2000), followed by the Guidelines for Drinking Water Quality in 1984 (WHO, 1984). The EU and the USA have developed and over the years expanded legislation which controls the release and the concentrations of certain chemicals. The European Drinking Water Act of 1980, for example, does not allow pesticide concentrations to exceed 0.1 $\mu g\ L^{-1}$ for individual substances or 0.5 $\mu g\ L^{-1}$ for total pesticides (Trinkwasserverordnung, 2000).

Enforcement of this legislation is only possible through reliable monitoring of the environment for the presence of substances which adversely affect the health of humans and ecosystems. Conventional analytical techniques are continually being improved and have reached high accuracy. However, they can only detect concentrations of known substances, while being unable to indicate the toxicity of a sample (Dennison and Turner, 1995).

Biotests, on the other hand, can reveal the effects of chemicals on organisms (or parts of organisms). Moreover, they can show the cumulative effect of all present substances, even of those which are unknown (e.g. breakdown products). They can also reveal interactive effects (antagonistic or synergistic) of chemicals (Gunkel, 1994). This enables the determination of the potential risk of the substance(s) (Nusch, 1993).

Due to these advantages over conventional analytical techniques, several biotests are already being employed routinely and have been included in OECD-Guidelines and DIN-Norms (Brüggemann and Steinberg, 1995). Nevertheless, a sharp increase in the number of publications concerning the application of biomarkers (Benford *et al.*, 2000) and

biosensors (Dennison and Turner, 1995) was evident in recent years. This indicates the persisting need of biotests for environmental monitoring (Markert, 1994).

## 1.1 Pesticides

Pesticides constitute a group of substances, which are known to produce adverse effects on humans and the environment. The United States Environmental Protection Agency (EPA) defines a pesticide as "any substance or mixture of substances intended for preventing, destroying, repelling, or mitigating any pest" (EPA, 1999). They are divided into groups, according to their target organism; the three major ones being insecticides, herbicides, and fungicides (PMRC, 2000). They confer large economic benefits by increasing yields and stabilising food production. In some areas, crops can only be grown through the use of pesticides (Seiber and Ragsdale, 1999).

However, since pesticides are potent toxic substances, side-effects can have significant negative impacts. Pesticides acting upon non-target organisms, for example, can affect biodiversity. Furthermore, residuals in water, air, and food can have adverse impacts on public health (OECD, 1997). Monitoring the environment for the presence of pesticides is crucial, while knowledge of their mode-of-action and behaviour is essential to understand and react to their effects on human health and the environment (Cook *et al.*, 2000).

Pesticides have various modes-of-action (Table 1.1) more or less specific to the target organisms. Most insecticides, for example, affect one of five biological systems in the target organisms: the nervous system, cuticle synthesis, the endocrine system, water balance, and energy production (Valles and Koehler, 2000). Herbicides, for the most part, affect inherent processes in plants and not mammals or insects - the inhibition of photo-synthesis being the best example. Moreover, the biosynthesis of carotenoids, amino acids or lipids can be affected (Kirkwood, 1991). The mode-of-action of fungicides includes the inhibition of various substances, such as chitin, melanin, proteins, or sterol biosynthesis, which disrupts membrane function (Hewitt, 1998).

The distinct sites of action and differences in specificity not only increase the range of pests to be controlled but also reduce the risk of pest resistance towards the pesticides (Cook *et al.*, 2000). However, the continuous development of new products with different modes-of-action increase the difficulty of the detection of contaminating pesticides. Therefore, new tests have to be developed in parallel to the pesticides to protect human health and the environment.

Table 1.1: Examples of insecticides, herbicides and fungicides which affect specific target sites (Bt: *Bacillus thuringiensis*).

| **Target Site** | **Insecticide** | **Herbicide** | **Fungicide** |
|---|---|---|---|
| Cell wall | - | Benzamides, Nitriles | Polyoxins, Tricyclazole |
| Chitin synthesis | Benzoylphenyl ureas | - | Polyoxins |
| Membrane | d-endotoxins (Bt) | Dinitrophenols | 1,2,4-Triazoles, Imidazoles |
| Lipid synthesis | - | Benzofurans, Thiocarbamates | Validamycin |
| Amino acid and Protein synthesis | - | Imidazolinones, Sulfonylureas | Kasugamysin |
| RNA synthesis | - | - | Hydroxypyrimidines, Phenylamides |
| Pigment synthesis | - | Pyridazinones, Triazoles | Tricyclazole |
| Microtubules | - | Carbamates, Dinitroanilines, Pyridazines | Benzimidazoles, Phenylcarbamates |
| Energy metabolism | Rotenone, Sulphonamid | Dinitrophenols | Nitrophenol derivatives |
| Hormones | Methoprene | Benzoic acids, Phenoxy-carboxylic acids | - |
| Nervous system | Amidines, Carbamates, Organophosphates, Pyrethroids | - | - |
| Photosynthesis | - | Amide, Nitriles, Triazines, Uracils, Ureas | - |
| References | Bloomquist, 2000; Vallas and Koehler, 2000 | Kirkwood, 1991; HRAC, 2000; Hartzler, 2001 | Hewitt, 1998 |

## 1.2 The Mitochondrial Electron Transport Chain

One site of pesticide action is the production of energy (Kirkwood, 1991; Hewitt, 1998; Bloomquist, 2000). During oxidative phosphorylation high-energy electrons traverse the mitochondrial electron transport chain, oxidising reduced coenzymes and finally producing water as they are transferred to oxygen (Babcock, 1999). The built-up proton motive force is used to drive the synthesis of ATP (Siedow and Umbach, 1995).

The electron transport chain consists of four integral multiprotein complexes (Figure 1.1). Complex I (NADH: ubiquinone oxidoreductase) oxidises NADH generated in the mitochondrial matrix via the citric acid cycle. The resulting electrons are passed to ubiqui-

none, while protons are translocated across the inner membrane. Complex II (succinate: ubiquinone oxidoreductase) contains succinate dehydrogenase, an enzyme of the citric acid cycle, which catalyses the oxidation of succinate to fumerate. The electrons are again transferred to ubiquinone. However, no proton transport occurs. Complex III (ubiquinone: cytochrome oxidase) oxidises the ubiquinone reduced by complexes I, II, and two NADH dehydrogenases. The electrons are passed to cytochrome c, while protons are transported across the membrane (Siedow and Umbach, 1995). Complex IV (cytochrome oxidase) oxidises cytochrome c, using the electrons to reduce oxygen to 2 $H_2O$ molecules to clear the mitochondrial chain of low energy, spent electrons (Babcock, 1999). The energy released in this process is coupled to the translocation of protons to contribute to the chemiosmotic gradient (Saraste, 1999). The $F_oF_1$ synthase allows the movements of the protons back across the membrane, using the energy to convert ADP and $P_i$ to ATP (Siedow and Umbach, 1995).

Plants have two paths for the transport of electrons from ubiquinone to oxygen. The first is the cytochrome pathway (Figure 1.1, blue arrows), as just described. The second is the cyanide resistant, alternative pathway (Figure 1.1, red arrow), which is comprised of a single protein, the alternative oxidase, on the inner mitochondrial membrane (Ordentlich *et al.*, 1991; Maxwell *et al.*, 1999). The engagement of the alternative pathway depends on the amount of protein present, the degree of ubiquinone reduction, and the activity of the alternative oxidase. The latter is influenced by the redox state of the regulatory sulfhydryl/disulfide bond of the enzyme, being inactive in the oxidised state and active in the reduced form (Wagner and Krab, 1995). Organic acids (e.g. pyruvate, succinate or malate) can activate it by lowering the apparent $K_m$ of the alternative pathway for reduced ubiquinone (Ribas-Carbo *et al.*, 1995), increasing its reactivity towards ubiquinone (Ribas-Carbo *et al.*, 1997). Unlike the main pathway, the alternative pathway is non-phosphorylating, as the electrons bypass two of the three proton translocation sites (Maxwell *et al.*, 1999) (Figure 1.1). The question therefore arises, why this apparently energetically wasteful pathway has been maintained through evolution, especially in view of the alternative oxidase being able to compete directly with the main pathway for electrons (Siedow and Umbach, 1995; Gonzàlez-Meler *et al.*, 1999).

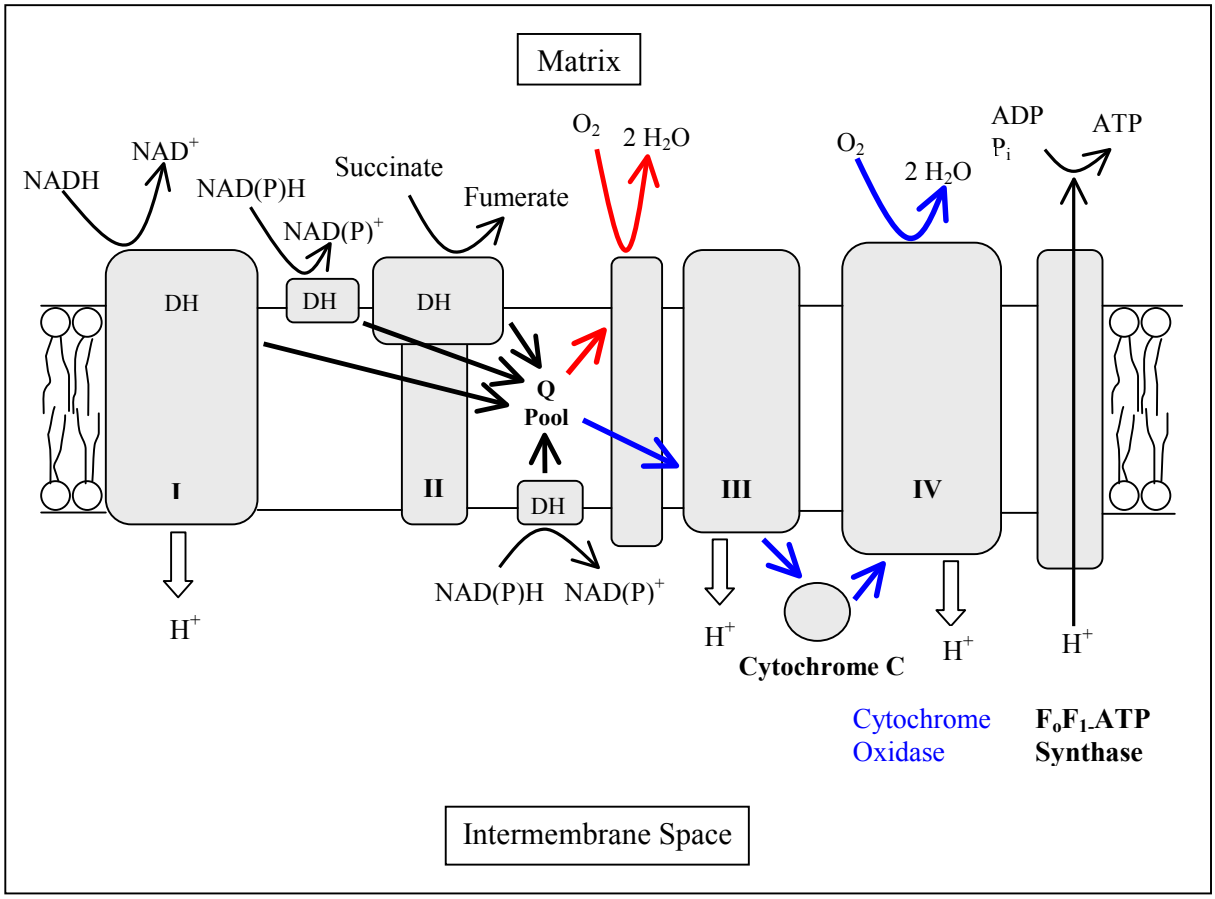



Figure 1.1: Organisation of the plant electron transport chain in the inner mitochondrial membrane, containing four integral multiprotein complexes (I-IV) (modified after Siedow and Umbach, 1995). The Q Pool is a pool of ubiquinone which diffuses freely within the inner membrane. It accepts electrons from the four dehydrogenases (DH) and transfers them to either complex III (blue arrows: cytochrome pathway) or the alternative oxidase (red arrows: alternative pathway).

The "only obvious physiological function" (Sluse and Jarmuszkiewicz, 1998) of the alternative oxidase is recognisable in specialised thermogenic tissues of plants. The thermogenic voodoo lily, for example, uses the heat to volatilise compounds to attract insect pollinators (Moynihan *et al.*, 1995). In *Symplocarpus*, the development of inflorescence and pollination, even at subzero temperatures in the environment, is guaranteed by long-lasting heat production (Meeuse, 1975). However, the presence of the cyanide-resistant, alternative pathway is not restricted to thermogenic plants. Apart from vascular plants, algae, fungi and some protists have also been found to contain the alternative pathway (Moynihan *et al.*, 1995). Therefore, the cyanide-resistant pathway has to play other physiological roles besides thermogenisis.

The role of the alternative oxidase in non-thermogenic tissues are being increasingly better understood (Sluse and Jarmuszkiewicz, 1998). It has been shown to be involved in

the antioxygen defence of plant mitochondria. Reactive oxygen species (ROS), such as superoxide, $H_2O_2$, and hydroxyl radicals, can cause oxidative damage of the inner mitochondrial membrane, leading to the impairment of the mitochondrial function (Kowaltowski *et al.*, 1998; Kowaltowski, 2000). They are readily removed under physiological conditions through a defence mechanisms of antioxidants (Leipner *et al.*, 2000; Munné-Bosch and Alegre, 2000) and antioxidant enzymes, such as catalase 3 and peroxidase (Prasad *et al.*, 1994; Zeng *et al*, 1994; Badiani *et al*, 1997; Prasad, 1997). The alternative oxidase plays its part in the antioxigen defence by reducing the amount of ROS formation (Wagner and Krab, 1995; Popov *et al.*, 1997).

Under pathological conditions, on the other hand, the antioxidant system may be defective or ROS generation can increase. Kowaltowski (2000) reported that the mitochondria are the main generation site for reactive species in most cells. ROS formation can result from an over-reduction of the mitochondrial respiratory chain components (Vanlerberghe and McIntosh, 1996; Maxwell *et al.*, 1999). This over-reduction stems from the saturation of the cytochrome pathway with electrons through the accumulation of organic acids or the restriction of the pathways' activity (Wagner and Krab, 1995; Vanlerberghe and McIntosh, 1996). Low ADP availability (Popov *et al.*, 1997), inhibition (Wagner and Krab, 1995) or stress induced physical changes of membrane components (Siedow and Umbach, 1995) have been reported to restrict the main pathway. The electron flow through the alternative pathway will maintain or return the ubiquinone reduction to a stable level, decreasing (the potential of) ROS formation (Wagner and Krab, 1995) by removing excess reducing equivalents which could reduce $O_2$ to $O_2^{-\bullet}$ (Popov *et al.*, 1997).

Apart from maintaining stable levels of ubiquinone reduction, the alternative pathway also seems to avoid ROS formation in other ways. Complex III is a step of the respiratory chain, which produces $O_2^{-\bullet}$ at a high protonic potential, being eluded as it is bypassed (Cadenas *et al.*, 1977). Popov *et al.* (1997) reported on the function of the alternative oxidase in respiratory protection, as it lowers the intra-mitochondrial oxygen level. Therefore by maintaining safely low levels of oxygen and its one-electron reductants, the alternative oxidase seems to play an important role in the antioxygen defence in mitochondria (Popov *et al.*, 1997; Braidot *et al.*, 1999).

## 1.3 Biological Monitoring

Increasing levels of pollution around the world call for more intensive monitoring of environmental quality. Biomonitoring (as opposed to chemical monitoring) is of special interest since it can be used to make predictions of the effects of contaminants on organisms or communities (Gunkel, 1994).

Biomonitoring is an "extended programme of surveys undertaken in order to provide a time series, to ascertain the extend of compliance with a predetermined standard of the degree of deviation from an expected norm" (Goldsmith, 1990) using bioindicators. These bioindicators are assessed on organisms, populations, communities, or parts of organisms, which react towards environmental stressors by changing their biochemistry and/or behaviour, or accumulate substances (Zimmermann, 1996).

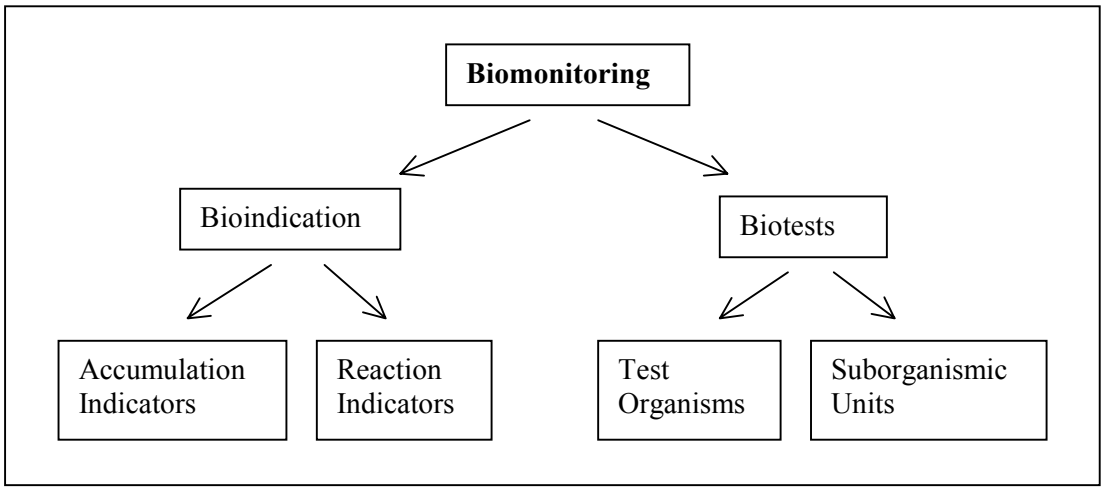



Figure 1.2: Biomonitoring strategies (Zimmermann, 1996; Wetzel, 1998).

Biomonitoring can be divided into two strategies: bioindication and biotests (Figure 1.2). Bioindication may involve indicators with specific accumulation or reaction patterns for certain chemicals (Wetzel, 1998) (Chapter 1.3.1). Biotests, on the other hand, are standardised techniques employing organisms ('test organisms') or part of organisms ('suborganismic units') to measure the biological effects of substances (Zimmermann, 1996) (Chapter 1.3.2).

To assess the degree of contamination of the environment and the (potential) effects of these substances on organisms, populations, communities, or part of organisms, several parameters can be measured at various levels of biological organisation (Table 1.2) (ESD, 2000). These parameters are employed in bioindication, as well as the biotests, as described later in this chapter.

Table 1.2: Parameters of stress response measured at various levels of organisation (modified after ESD, 2000)

| **Level of Organisation** | **Level of Assessment** | **Stress Responses** |
|---|---|---|
| Sub-organismic | Biochemical | Detoxification Enzymes, Bile Metabolites, DNA Integrity, Stress Proteins, Antioxidant Enzymes |
| | Physiological | Creatinine, Transamin. Enzymes, Cortisol, Triglycerides, Steroid Hormones |
| Individual | Histopathological | Necrosis, Macrophage Aggregate, Parasitic Lesions, Functional Parenchyma, Carcinomas |
| | Bioenergetic | Growth, Total Body Lipid |
| | Reproduction | Organo-Indices, Condition Factor |
| Population | Growth | Abundance, Reproductive Integrity |
| | Structure | Size and Age Distribution, Sex Ratio |
| Community | Structure | Richness, Intolerant Species, Food-Web Alterations, Trophic-Level Relationships |

Biomonitoring at the different levels of organisation varies in its ecological relevance of the measured responses, as well as the time scale in which the responses become evident. Community level biomonitoring provides information on the magnitude and ecological effects of stressors on the ecosystem (Gunkel, 1994). The measured responses are the least sensitive to contaminant stressors, only becoming evident in the long term. Biomonitoring using sub-organismal responses, on the other hand, has a low ecological but a high relationship-to-cause relevance. They are more sensitive to stressors and can be determined within shorter time scales (Shugart *et al.*, 1992). If lower-level responses (e.g. sub-organismal) are calibrated to higher-level responses (e.g. at the population/community level), the former can be used effectively in ecological risk assessment (Figure 1.3) (ESD, 2000).

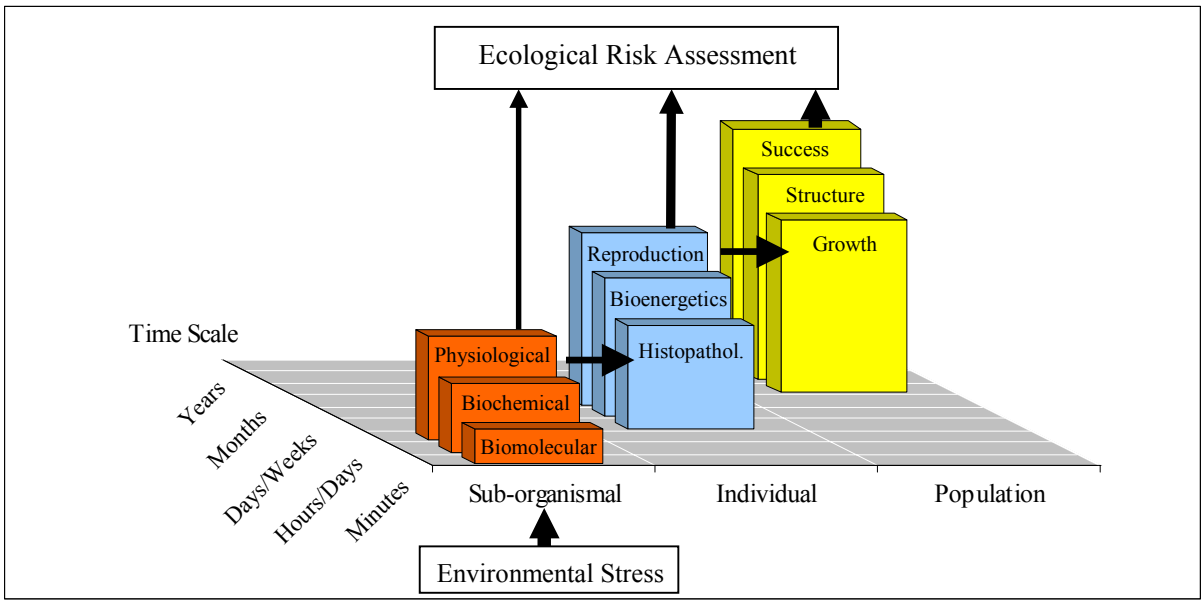



Figure 1.3: Responses to environmental stress at different levels of organisation (modified after ESD, 2000)

### *1.3.1 Bioindication*

Bioindication involves the use of reaction and accumulation indicators (Figure 1.2). Reaction indicators point out the presence of a toxicant as it reacts towards it by changes in the biochemistry (Zimmermann, 1996). All effects of toxicants originate in chemical processes at the molecular level (Fossi *et al.*, 1994). At the sub-organismal level, biomarkers can be used for biomonitoring purposes. Biomarkers are "parameter[s] which can be measured in a biological sample, and which provide information of an exposure, or on the actual or potential effects of that exposure in an individual or in a group" (Benford *et al.*, 2000). Biomarkers of exposure indicate the exposure of an organism towards a contaminant. These either include the substances themselves or their metabolites, as long as they are specific of the exposure of interest. In other words, the specific substance (or metabolite) may not be derived by any other means than through the exposure of the substance of interest, and all individuals must be able to produce the measured metabolite (Benford *et al.*, 2000). Fossi *et al.* (1999) reported on benzopyrene monooxygenase activities in marine mammal skin biopsy specimens as early indicators of exposure to endocrine disrupting organochlorines (Table 1.3). Similarly, the synthesis of vitellogenin in male fish results from their exposure to estrogenic compounds (Sumpter and Jobling, 1995; Kime *et al.*, 1999). Biomarkers of exposure may, therefore, become relevant outside the laboratory to carry out risk assessment. They cannot, however, predict the toxicity of substances – unlike biomarkers of effect (Benford *et al.*, 2000).

To determine biomarkers of effect it is necessary to have established the mechanistic causal pathway, which links the measurements of exposure, intermediate effects and the final outcome (disease) (Bennett and Waters, 2000). DNA-adducts, for example, have been shown to be key elements in the initiation of chemical carcinogenesis. They are formed by PAHs (polycyclic aromatic hydrocarbons) and other genotoxic substances by binding covalently to the DNA (Gram, 1985). Such DNA alteration can lead to irreversible changes to the DNA molecule, resulting in expression of chromosomal aberrations and oncogene activation (Shugart, 1994).

If the impact of toxicants at the molecular or biochemical level exceeds the compensatory responses, it passes to successively higher levels of organisation. It is therefore of importance to also monitor responses at the population and community levels (Fossi *et al.*, 1994) (Table 1.2). The Biotic Indices are used to classify the degree of pollution according to the tolerance of indicator species to pollutants. The measures include indices such as richness, pollution tolerance, trophic level present, and abundance (Table 1.3) (NCSU, 2000). The US Environmental Protection Agency (EPA) Rapid Bioassessment Protocol for Use in Streams and Rivers, for example, uses community diversity to determine water quality. Pollution may be indicated by the absence of pollution sensitive benthic macroinvertebrates (Ephemeroptera, Plecoptera, and Trichoptera) and dominance of pollution-tolerant groups (Oligochaetes or Chironomids). In general, low richness can indicate impairment (Plafkin *et al*, 1989). Apart from the just described reaction indicators, accumulation indicators are also of importance in biomonitoring.

Accumulation indicators are different from the bioindicators described so far, in that no response towards the contaminants is determined. Their value lays in their ability to accumulate substances from their surrounding, sequestering them in their bodies. Analyses of the tissues allow an indirect estimate of the environmental concentrations. The increased contaminant concentrations lead to a higher accuracy, sensitivity, and reproducibility of the measurements (Markert, 1994). These indicators can be sentinel organisms, such as plants (e.g. hazel, sycamore (Little, 1977), grasses (Ho and Tai, 1988), filter feeders, isopods, and seaweeds (e.g. *Fucus* spp.) (Kennish, 1992; Phillips and Rainbow, 1993). Accumulators can also be introduced into the environment. So-called 'active' indicators have the main advantage of accumulating substances over a known time period. Goodman and Roberts (1971) and Little and Martin (1974) suspended moss (*Hypnum* and *Sphagnum*, respectively) in nylon bags. Accumulation indicators have proved invaluable,

rapid and inexpensive means of monitoring environmental pollution (Little and Martin, 1974).

Table 1.3: Examples of bioindicators used for bioindication and biotests to determine the presence and/or effects of pesticides (AChE: Acetylcholinesterase).

| | **Bioindicator** | **Parameter** | **Pollutant** | **Reference** |
|---|---|---|---|---|
| Bioindication | Fish | AChE Inhibition | Carbamates, Organophosphates | Haubruge *et al.*, 1997 |
| | Marine Mammals | Benzopyrene Monooxygenase Activity | Endocrine Disrupting Organochlorines | Fossi *et al.*, 1999 |
| | Benthic Macroinvertebrates | Richness, Abundance | Heavy Metals | NCSU, 2000 |
| Biotest (Test Organisms) | Daphnia | Growth and Reproduction | Endosulfan | Fernandez-Casalderrey *et al.*, 1993 |
| | | | Lindane | Ferrando *et al.*, 1995 |
| | Daphnia | Population Dynamics | Endosulfan | Barry, 1996 |
| Biotest (Suborganismic) | Thylakoid Membranes | Fluorescence | Diuron | Schnabl *et al.*, 1999 |
| | AchE, Choline oxidase | AChE Inhibition | Carbamates, Organophosphates | Dennison and Turner, 1995 |

### *1.3.2 Biotests*

Several biotest have been standardised by the OECD and the International Organisation for Standardization (ISO) and are routinely being employed to assess the effects of specific substances. Toxicity tests listed in the OECD-Guidelines include the algae growth inhibition test, the acute immobilisation test and reproduction test using *Daphnia* sp., life-cycle tests on fish, and the AMES-Test (*Salmonella* mutagenicity) (Table 1.3) (Brüggemann and Steinberg, 1995; Wetzel, 1998). All of these techniques employ whole organisms (Henry, 2000).

Suborganismic units are incorporated in biosensors, a special type of biotest. Biosensors are "analytical devices, which incorporate a biological component and a transducer" (Holme and Peck, 1994). Transducers convert the detection by the biological components into electrical signals. The possible biological components fall into two categories: biocatalysts and bioreceptors. The former involves binding of the substance, followed by a chemical reaction and the release of products. They include enzymes, microbioal/plant/

animal cells or sub-cellular organelles. For bioreceptors, binding is non-catalytic and irreversible, as seen with antibodies or cellular membrane receptors. It is the properties of these biological components, which give biosensors their specificity (Holme and Peck, 1994).

This specificity can range from being very narrow to encompassing an array of substances. Some biosensors have been developed to detect specific chemicals. Employing alkaline phosphate labelled anti-antibodies to bond to atrazine antibodies, for example, can indicate atrazine. A fluorescent product is yielded after the addition of an enzyme substrate. The detection limit lays at $10^{-2}$ pmol atrazine $mL^{-1}$. (Scheper and Müller, 1994). Other biosensors can detect groups of substances by making use of their chemical properties. Organophosphate and carbamate insecticides inhibit acetylcholinesterase (AChE) and choline oxidase (Table 1.3). The former enzyme yields choline from acetylcholine, while choline oxidase oxidises choline to betain and hydrogen peroxide. The latter can be measured amperometrically (Marty *et al.*, 1992). Biosensors can also employ the actual target site of chemicals. A newly developed biosensor uses lyophilised thylakoid membranes to detect the presence of herbicides, which interact with the photosynthetic electron transport (Schnabl *et al.*, 1999). As the electron transport is inhibited, the energy is released as light. This fluorescence can be measured, reaching a detection limit of 0.1 µg Diuron $L^{-1}$ (Walz, 2000). The biological component and its related specificity, therefore, clearly govern a biosensor's utilisation.

Determining the degree of contamination of an environmental sample using biotests often involves taking samples into the laboratory. The greatest advantages of biosensors, therefore, are their small size, the reduced need for sample preparation and their fast response time, making them ideal for use in the field (Dennison and Turner, 1995).

Over the last decade it has increasingly become evident that no single test can meet all needs or answer all questions (Henry, 2000). As indicated above, the effects of contaminants vary between the levels of biological organisation and time scales of response (ESD, 2000). Moreover, the sensitivity of different species, as well as of individuals, towards contaminants will vary (Gunkel, 1994). The presence of confounding factors will further increase the difficulty of assessing cause-effect-relationships (NSCU, 2000). Many tests are therefore needed and should be selected and combined with others according to the contaminants of interest and the questions to be addressed (Nusch, 1993; Henry, 2000).

## 1.4 The Protoplast Biosensor

A biosensor, which has been developed in this Institute is the protoplast biosensor. It uses mesophyll protoplasts of higher plants as the biological component to detect harmful substances, which affect respiration and photosynthesis. These effects can be determined as changes in the rates of oxygen-use and -evolution, using a Clark oxygen electrode, which measures dissolved oxygen in the protoplast/sample mixtures.

The Clark oxygen electrode (Figure 2.1) is an amperometric transducer with two electrodes: a working electrode (cathode), through which the potential is applied and a reference electrode (anode), both of which are connected by an electrolyte solution. Oxygen diffuses through the membrane, which covers the cathode, being reduced at the latter, resulting in a negative potential.

Cathode reaction: $O_2 + 2\ H_2O + 2\ e^- \rightarrow H_2O_2 + 2\ OH^-$

$H_2O_2 + 2\ e^- \rightarrow 2\ OH^-$

The spent electrons are replaced by the anode reaction, made possible by the anode residing in an electrolyte (e.g. potassium chloride) (Figure 1.4).

Anode reaction: $4\ Ag \rightarrow 4\ Ag^+ + 4\ e^-$

$4\ Ag^+ + 4\ Cl^- \rightarrow 4\ AgCl$

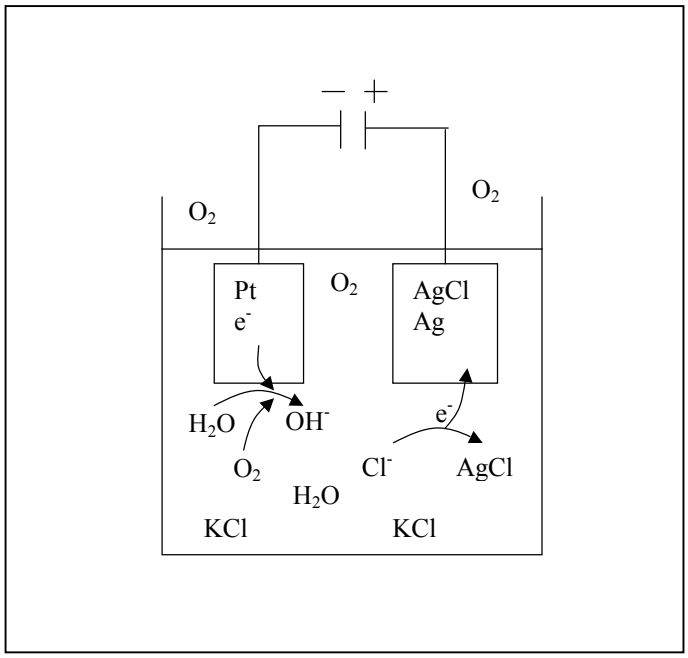



Figure 1.4: Schematic presentation of the reactions on which the Clark oxygen electrode is based (Pt: platinum working electrode (cathode); Ag/AgCl: Silver/silver chloride reference electrode (anode); KCl: Potassium chloride electrolyte solution).

A current proportional to the number of oxygen molecules within the protoplast/sample mixture is therefore produced between the cathode and the anode. An interface provides the polarising voltage to the electrodes, amplifies the low-level signals, makes the A/D conversion and finally sends the resulting data to a computer (Holme and Peck, 1994; Strathkelvin, 1999).

In order to determine the amount of dissolved oxygen within a sample, the influences of external factors have to be minimised. Stirring the sample during the measurement is necessary to avoid the formation of concentration gradients. Furthermore, a water jacket surrounds the cuvette containing the sample. The water within it is kept at a constant temperature. This is important since oxygen solubility in water, as well as its diffusion through the membrane, are temperature dependent. Solving these problems has resulted in a sensitive and reliable transducer used worldwide in areas ranging from physiology and pharmacology to ecotoxicology, where it is used to measure dissolved oxygen in various media, such as blood, mitochondria or enzyme preparations, microbial suspensions or even larger animals like mussels (Strathkelvin, 1999).

As mentioned in the previous section, it is the biological component which gives a bio-sensor its specificity. Mesophyll protoplasts of higher plants were chosen for various reasons. First of all, they present model systems of the effects of substances on metabolic processes of higher plants, since the effects are determined at the cellular level (Overmeyer, 1993). Furthermore, since protoplasts, as opposed to cells, have had their cell walls removed, their plasma membranes are more accessible and may be crossed more easily. As a result, substances can elicit their effects at shorter time scales and the sensitivity is increased (Overmeyer *et al.*, 1994a).

The viability of the protoplasts in the presence of environmental samples is determined from their rates of oxygen-use and -evolution. The production of $O_2$ (i.e. photosynthesis) is measured in the light, while oxygen-use is determined in the dark (Overmeyer *et al.*, 1994a). Lindner (1993) investigated the effects of environmental factors such as temperature, pH, and ions (e.g. nitrate, phosphate, chloride, calcium, potassium, magnesium) on the viability of protoplasts. Only temperature and pH, as well as light intensity and osmolarity of the test medium affected it. All of these factors, however, can be controlled. The presence of the investigated ions in samples did not show an effect, therefore presenting no problem during measurements of complex environmental samples.

The protoplast biosensor has been employed for the detection of ecotoxicological substances in different media. Waste water from a sewage treatment plant was screened for contaminants (Schnabl and Zimmermann, 1989). Rain water was tested for atmospheric pollution with herbicides, which act upon the photosynthetic system (Overmeyer *et al.*, 1994b). Overmeyer *et al.* (1994a) developed an on-line monitoring system to continually measure water quality of surface waters, using the river Rhine as an example. Such a system could be employed as an early warning system to detect and react quickly to pollution spills (for example near industrial plants).

However, two problems exist with using protoplasts as biological components of a biosensor: loss of viability and cell wall regeneration. Within several hours after isolation protoplasts synthesise new cell walls (Upadhya, 1975; Galbraith, 1981). This means the loss of the essential property of an unencumbered and easily accessible plasma membrane (Grout, 1995).

The greatest problem, however, is the loss of protoplast viability. Within 48 h protoplasts showed degradation of chlorophyll a and b pigments, resulting in a continuous reduction in light-dependent oxygen-evolution. Changes in key enzymes, such as neutral protease and ribulose biphosphate carboxylase (RuBPcase) were also determined. An increase in ethane evolution indicated cellular disorder and the loss of compartmentalisation (Schnabl and Zimmermann, 1989). These symptoms could be delayed for a week by immobilising the protoplasts in alginate matrices (Lindner *et al.*, 1992).

However, the immobilisation also presented its difficulties. Bacterial and fungal infections occurred within one week, even after the addition of antibiotics (Lindner *et al.*, 1992). Employing protoplasts of sterile sunflower plants did not improve the situation, since those protoplasts contained very low amounts of chlorophyll. This constituted an extremely undesirable side effect when measuring the rate of oxygen-evolution, since the number of protoplasts had to be increased. As a result, more protoplasts had to be isolated. Moreover, when employing immobilised protoplasts the magnetic stirrers in the cuvettes were blocked (Lindner, 1993). Yet, possibly the gravest problem was the large mean variation of oxygen measurements using immobilised protoplasts, due to their uneven distribution in the viscous alginate (Overmeyer, 1993). Therefore, a new method had to be developed to maintain the viability of the protoplasts over a longer time period, making them available as biological components for the biosensor.

## 1.5 Freezing

As freezing can preserve cells and their constituents, it might be used to prevent the loss of protoplast viability and the regeneration of a cell wall. However, freezing can also be lethal to most organisms (Mazur, 1970). To avoid fatal injuries to the protoplasts it is important to determine what kind of damage occurs during a freeze/thaw cycle. Subjecting cells to subzero temperatures initially leads to the supercooling of the cell (or protoplast) interior. Ice nucleation occurs in the suspending medium at a temperature, which depends on the freezing point of the solution and the presence of ice-nucleating agents. As the water freezes, the solutes are excluded from the ice, concentrating the partially frozen solution. To reach an osmotic equilibrium between the outside and inside, water leaves the cell, resulting in cellular dehydration and concentration of intracellular solutes. The cooling rate influences this process: During slow cooling more time is available for water to leave the cell, while at rapid cooling rates extensive supercooling occurs eventually forming intracellular ice through nucleation or seeding of the supercooled solution. The manner of equilibration is primarily determined by the plasma membrane, which assumes a central role in the behaviour of a cell during the freeze/thaw cycle (Steponkus, 1984).

Various stresses are encountered between the time in which cells are first exposed to extracellular ice nucleation and the time they are returned to post-thaw conditions. These include mechanical stresses incurred by the plasma membrane during osmotic contraction and expansion, and chemical stresses due to solute concentration and dehydration (Dowgert and Steponkus, 1984). This indicates that freezing causes a multitude of stresses and injury is a result of their interaction (Steponkus, 1984).

### *1.5.1 The Role of the Plasma Membrane in Freezing Injury*

Experiments on isolated protoplasts revealed that the plasma membrane is disrupted during the freeze/thaw cycle, resulting in a spectrum of alterations in the semi-permeable characteristics. Injury can manifest itself as *(1)* expansion-induced lysis, *(2)* loss of osmotic responsiveness, *(3)* altered osmotic behaviour, and *(4)* intracellular ice formation (Steponkus, 1985).

Expansion-induced lysis occurs during warming and thawing of the suspension medium when the decreasing osmolalities of the medium lead to the osmotic expansion of protoplasts (Steponkus, 1984). Steponkus *et al.* (1983) showed that lysis occurred during warming before the original volume and surface area were regained, thus calling it

'expansion-induced lysis' (Figure 1.5 (f)). The basis for this type of injury is, however, laid during freezing. Meryman and Williams (1985) raised the "minimum cell volume" theory postulating that "for every cell there is a volume beyond which it cannot be reduced" without injury. Therefore, as water is removed below a certain volume (Figure 1.5 (c)) irreversible changes occur (Clegg *et al.*, 1982). During cooling, the plasma membrane revealed endocytotic vesiculation, i.e. deleted material in cytoplasmic vesicles. This material was no longer readily available for reintegration into the plasma membrane during subsequent expansion. Since endocytotic vesiculation requires breakage and fusion of the plasma membrane, it is predisposed to mechanical failure and can lead to intracellular ice formation through seeding of the supercooled cytoplasm (Dowgert and Steponkus, 1984).

Loss of osmotic responsiveness is another manifestation of injury. Protoplasts remain inactive during warming, because of alterations of the plasma membrane in the contracted state, therefore failing to expand. It is possible that osmotic inactivity and intracellular ice formation are two different manifestations of plasma membrane mechanical breakdown. The occurrence of one or the other depends on whether or not the protoplasts achieved osmotic equilibration before the membrane is damaged (Steponkus *et al.*, 1983). However, little is known about the causes of the loss of semi-permeable characteristics (Steponkus, 1984).

Altered osmotic behaviour is indicated by the volume of protoplasts being less after thawing than before the freeze/thaw cycle (Figure 1.5 (g)). This suggests a transient loss of intracellular solutes or a 'leakiness' of the membrane. However, since no lysis occurs, it is considered a sublethal manifestation of injury (Steponkus, 1984).

Intracellular ice formation, on the other hand, is always lethal to cells due to the physical sharing of intracellular membranes and other structures (McKersie, 1996a). Damage is caused mainly during thawing, as ice crystals grow through recrystallisation (Figure 1.5 (h)) (Farrant *et al.*, 1977; Merymann and Williams, 1985). As mentioned previously, intracellular ice formation predominates at rapid cooling rates (Figure 1.5 (e)) and is considered to be a consequence of membrane disruption, as it allows the supercooled intracellular solution to be seeded by the extracellular ice (Dowgert and Steponkus, 1984). An intact plasma membrane presents an effective barrier to external ice crystals (Steponkus, 1985). It therefore seems that the mechanical failure of the plasma membrane plays an important part in the manifestation of freezing injury.

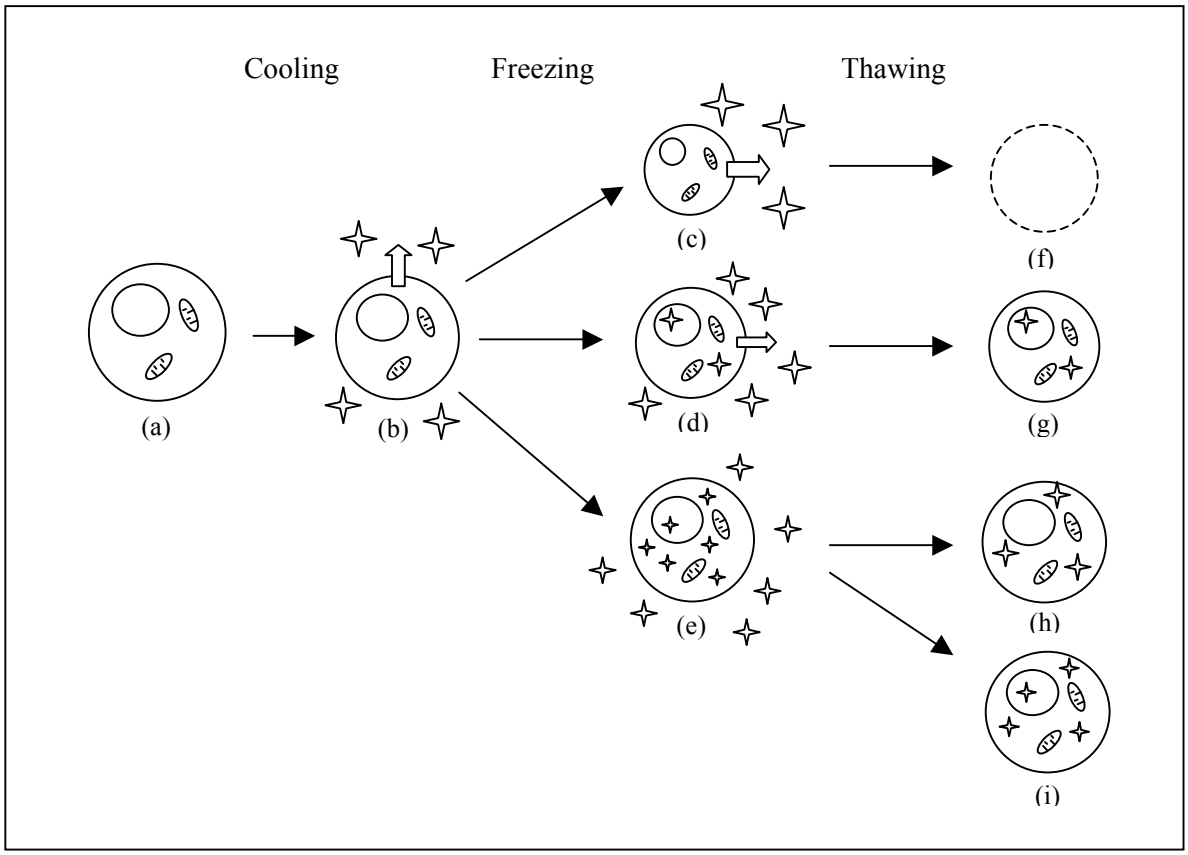



Figure 1.5: Events occurring during a freeze/thaw cycle (modified after Mazur, 1970).

(a) Untreated protoplast (PP).
(b) PP cooled to -5°C: cytosol is supercooled, ice formation occurs on the outside, water consequently leaves the PP to achieve osmotic equilibrium.
(c) PP after slow cooling: as ice crystals form extracellularly and the extracellular solution is increasingly concentrated, water leaves the PP, leading to PP shrinkage and dehydration. "Solution effects" become predominant due to the concentration of the PP contents. Cell attains osmotic equilibrium before the characteristic nucleation temperature is reached and is therefore not subject to intracellular freezing.
(d) PP after moderate cooling: not as much time to attain osmotic equilibrium before the characteristic nucleation temperature is reached. Thus, smaller ice crystals on the outside as under slow cooling conditions (c), some ice crystallisation inside the PP, some water leaves the PP, resulting in shrinkage.
(e) PP after rapid cooling: many small ice crystals, outside and inside the PP. Virtually no time for water to leave the cell, thus very little shrinking.
(f) PP after thawing: expansion-induced lysis (esp. in non-acclimated PP) before PP regains the original volume and surface area. May be prevented by acclimation of the plant/tissue/cell.
(g) PP after thawing: altered osmotic behaviour. PP volume is less after thawing than before freezing.
(h) PP after slow thawing: increasing ice crystal growth (recrystallisation) with increasing temperature.
(i) PP after rapid thawing: less recrystallisation than in (h), as less time is available for crystals to join. Less damage and higher chance of survival for PP.

Several stresses may be responsible for membrane damage. Dehydration changes the organisation of membrane lipids, leading to phase transitions. At physiological temperatures and hydration the phospholipid bilayer of biological membranes is in a liquid-crystalline state (Figure 1.6 (1)), allowing rotational and lateral movements of phospholipids and integral proteins within the bilayer. In the gel phase (Figure 1.6 (2)), on the other hand, the mobility of lipids is severely restricted and the packing of acyl chains is tighter (McKersie, 1996b). At conditions of low temperature or hydration phospholipids can undergo a phase transition into hexagonal$_{II}$ phase (Figure 1.6 (3)), forming long cylinders with the polar headgroups orientated into an aqueous core (Crowe *et al.*, 1983). This leads to a change in the association of integral and peripheral proteins with membrane lipids, altering electron transport processes and solute transport across the plasma membrane. Both would contribute to a long-term change in permeability and enhance leakage (McKersie, 1996b).

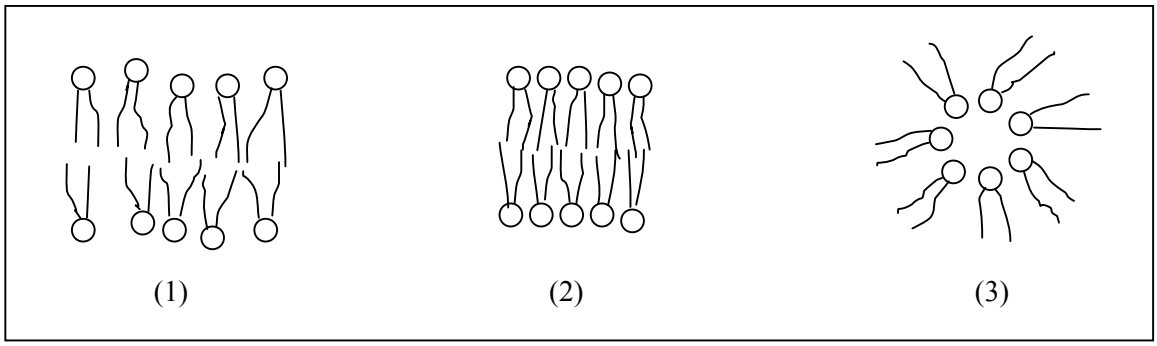



Figure 1.6: Model of phospholipid packing showing liquid-crystalline phase (1), gel phase (2) and hexagonal II phase (3) (after McKersie, 1996b).

Electrical perturbations can also affect membrane integrity. Freezing of aqueous electrolyte solutions leads to differential exclusion of ions from the ice, causing a potential difference across the ice interface. These potential differences may cause destabilisation and lysis of the plasma membrane (McKersie, 1996a).

The multitude of injury manifestation indicates that freeze/thaw injury is a multifaceted complex (Steponkus 1984). In view of the current knowledge of these injuries, Mazurs' statement (1970) that the "ability of a cell to survive freezing may depend more on protection of the cell surface than on protection of the cell interior" still seems to hold.

### *1.5.2 Cold-Acclimation*

A common hypothesis is that cells of cold-acclimated plants are protected during freezing by the accumulation of specific metabolites, which protect the cell during a freeze/thaw cycle. However, "it has proven very difficult to determine

- which of these metabolic changes are critical to the acquisition of freezing tolerance,
- which are simply adaptations to growth at low temperatures,
- which are injury responses,
- which are primary and
- which are secondary effects" (McKersie, 1996a).

Some generalisations are often observed:

a) The osmotic concentration increases with acclimation. A doubling of the internal solute concentration will decrease the extent of cellular dehydration by 50% (Steponkus, 1984). Major changes in the osmotic potential are ascribed to changes in sugars. These can depress the freezing point through intracellular osmolarity (Meryman and Williams, 1985), alter phase properties of membranes in the dry state, or preserve protein structure and function (McKersie, 1996a). Non-reducing sugars, especially trehalose, have been suggested to prevent dehydration-induced membrane fusion by acting as membrane spacers (Steponkus, 1984).

b) An inverse relationship between the water content and freezing tolerance seems related to the accumulation of starch and protein which are not osmotically active (McKersie, 1996a). Certain hardy plants appear to have the ability to prevent cell water from participating in osmotic events (Meryman and Williams, 1985).

c) Following cold acclimation, changes in sterol content and fatty acid unsaturation have been observed, as well as an increase in phospholipid-to-protein ratio (Steponkus, 1984).

d) A close relationship between soluble protein content and freezing tolerance has been determined (McKersie, 1996a).

The major distinction between non-acclimated and acclimated protoplasts is the temperature at which intracellular ice formation takes place. As the mechanical breakdown of the plasma membrane is the primary cause of seeding the supercooled cell interior, and cold-acclimation alters the characteristics of the plasma membrane to try and avoid its breakage during freezing, seeding occurs at lower temperatures in acclimated protoplasts (Dowgert

and Steponkus, 1983). Steponkus *et al.* (1983) revealed that those protoplasts formed exocytotic extrusions of plasma membrane during freezing, without membrane disruption. On deplasmolysis of the protoplasts the membrane material was reincorporated, avoiding expansion-induced lysis (Steponkus, 1984).

Generally, changes in membrane composition are responsible for differences in membrane behaviour (Dowgert and Steponkus, 1984). Cold acclimation increases the tolerance of the plasma membrane to mechanical stresses during hypertonic stress, large area deformations, and applied electrical fields. The molecular bases responsible for this increased tolerance, however, are largely unknown (Steponkus, 1984).

### *1.5.3 Cryoprotection*

Cryoprotectants are compounds, which protect biological systems from detrimental effects during the freeze/thaw cycle. Several of these chemicals have been reported after studying organisms which acquire tolerance to dehydration from freezing, drying, or exposure to hyperosmotic environments. These cryoprotectants include sugars, sugar alcohols, amino acids, betaine and glycerol (Meryman and Williams, 1985).

Many compounds showing different cryoprotective properties have been employed, singly or in combination, in numerous studies to prevent freezing or dehydration damage of cells. Meryman and Williams (1985) reported that glycerol and dimethyl sulfoxide (DMSO) lowered the rate of water diffusion out of the cell. Maintaining a greater amount of water within cells, leads to a smaller degree of cell volume change. Moreover, a higher amount of water also reduces the concentration of solutes in solution, preventing their accumulation to toxic levels (Finkle *et al.*, 1985; Hitmi *et al.*, 1997). The lower diffusion rate of water to the outside of cells can also lead to a reduction of the cooling rate required to obtain the right amount of dehydration (Meryman and Williams, 1985). Polyvinylpyrrolidone (PVP), in contrast, avoids intracellular ice formation through the depression of the freezing point (Finkle *et al.*, 1985). The protection of the membrane from breakdown has been reported for trehalose (Anochordoguy *et al.*, 1987; Leslie *et al.*, 1995; Mc Kersie, 1996a), sucrose (Leslie *et al.*, 1995; Mc Kersie, 1996a) and the amino acid proline (Anochordoguy *et al.*, 1987). Sucrose (Remmele *et al.*, 1997) and proline (Steponkus, 1984) have also been shown to increase the stability of proteins. Studies have indicated that a combination of cryoprotectants seems more effective at maintaining viability after freezing than employing a single compound (Hitmi *et al.*, 1997).

To complicate the choice of an adequate cryoprotectant even further, cryoprotectants can have toxic effects on the cells they should protect, as does, for example, DMSO. The extent of the damage depends on the concentration, the time and temperature of the exposure, as well as on the rate of addition and dilution of the compound (Farrant *et al.*, 1977). Moreover, Farrant *et al.* (1977) reported that the cryoprotective properties vary with cooling rate. This indicates the need to develop freezing protocols depending on the species of interest (Withers, 1985).

In summary, preserving cells and their constituents through freezing may be hampered by several difficulties. The processes of freezing damage and cold acclimation are not yet fully understood. Furthermore, various cryoprotectants exist, whose properties may change according to the methodology. Therefore, developing freezing protocols presents a challenging quest.

## 1.6 Aims of the Study

The protoplast biosensor has proved to be a useful tool to determine contaminants in environmental samples in the laboratory. These contaminants include respiratory toxins, which are not measurable using the thylakoid biosensor (Chapter 1.3.2). Yet, several problems still exist, which hamper the applicability of the biosensor in the field. One important difficulty is the loss of the protoplasts' activity within a relatively short time span.

It was, therefore, the aim of this study to develop a method to advance the applicability of the protoplast biosensor under field conditions. Three sub-aims were defined to achieve the objective of this study:

- The identification of an appropriate donor plant to provide mesophyll protoplasts.
- The development of a method to preserve the biosensor properties of the protoplasts over longer time periods, ensuring the availability of the biological units. In accordance, a procedure should be developed to increase the mechanical stability of the protoplasts.
- Evaluating the utilisation and applicability of the frozen protoplasts as biological units of the biosensor.

# 2 MATERIALS AND METHODS

## 2.1 Plant Material

### *2.1.1 Non-sterile*

The seeds of *Vicia faba* L *var*. 'Weißkernige Hangdown' and *Helianthus annuus* var. 'Albena, KWS' were sown into potting soil treated with 100°C water vapour. The plants were grown in a climate chamber under the following conditions:

| | |
|---|---|
| Temperature: | 20°C |
| Relative humidity: | 70-90% |
| Light: | 12 h white light |
| Light source: | 2.4 W $cm^{-2}$; lamp type Phillips HPL-N |

For cold acclimation, three weeks old *H. annuus* plants were transferred from the 20°C to a chamber with 8°C. The latter maintained climatic conditions similar to the former, with the exception of a 10 h light period.

To isolate mesophyll protoplasts the second and third leaves of *Vicia faba* were collected from two to three week old plants. In the case of *H. annuus*, the first or second leaves were collected after two or three weeks, respectively. Acclimated plants were kept at 8°C for three weeks. From these plants the third leaves were used for mesophyll protoplast isolation.

### *2.1.2 Sterile*

*Helianthus annuus* seeds were sterilised as described by Henn (1997). The plant growth medium contained 2.15 g $L^{-1}$ MS-salts (Murashige and Skoog, 1962), 10 g $L^{-1}$ sucrose, 8 g $L^{-1}$ agar and 0.6 g $L^{-1}$ MES (pH 5.7). Growth conditions were as follows:

| | |
|---|---|
| Temperature: | 20°C |
| Relative humidity: | 70-90% |
| Light: | 16 h white light |
| Light source: | Warm white: Osram L 58W/31-830, Lumilux Plus, and<br>Cool white: Sylvania F 58W/840 (184), Luxline plus |

Second leaves were collected in a flow cabinet from three week old plants.

## 2.2 Isolation of Mesophyll Protoplasts

To increase the yield of the mesophyll protoplasts (PP), as well as their vitality, the isolation procedures for both plants were optimised.

### *2.2.1 Vicia faba*

Firstly, two digestion procedures were compared:

1) Incubation at 30°C for 4 h (enzyme solution 1, Table 2.1);
2) Incubation for 15 h at 25°C, increasing the temperature to 30°C for 1.5 h (enzyme solution 2, Table 2.1).

Antibiotics were added to reduce the number of bacteria during the over-night incubation. The effect of two concentrations of kanamycin and cefotaxime (10 µg $mL^{-1}$ and 50 µg $mL^{-1}$) was compared with respect to the number of protoplasts isolated. Since the enzyme solution 2 (Table 2.1) lead to the greatest protoplast yield, it was used for all evacuolation experiments (Chapter 3).

To determine whether tocopherol (vitamin E) would increase the vitality of the isolated protoplasts due to its radical scavenging characteristics, 0 µM, 5 µM and 10 µM DL-α-tocopherol phosphoric acid ester disodium salt was employed

1) during the digestion procedure and
2) after clean-up (i.e. resuspension in 0.6M mannitol solution).

Leaves were collected and placed in a beaker containing tap water. After weighing the towel-dry leaves, the epidermis on the upper surface was sanded off (sandpaper: P1200). The leaves were then placed upside down in a petri dish containing enzyme solution. After the incubation (time period as indicated above), the enzyme-protoplast suspension was passed through a 200µm mesh and the protoplasts were sedimented off (50 mL test tubes, 200 g, 5 min, 20°C; Minifuge GL, Heraeus Christ GmbH, Osterode). The protoplasts pellet was resuspended in sucrose solution (Table 2.1). The suspension was then transferred to 10 mL test tubes, topped with 2 mL 0.6 M mannitol solution (Table 2.1) and centrifuged (325 g, 15 min, 20°C; Heraeus Minifuge GL) using a discontinuous gradient to separate the protoplasts of remaining cell fragments. Most protoplasts accumulated in the interface between the two solutions and were sucked off using a pipette with a cut tip. The protoplasts were washed once in 0.6M mannitol solution (10 mL tubes, 200 g, 5 min, 20°C; Heraeus Minifuge GL). Finally, the pellet was placed in a fridge to settle for one

hour before evacuolation or oxygen measurements. Protoplast numbers were determined (Fuchs-Rosenthal Haemocytometer) and resuspended in 0.6 M mannitol solution to a defined concentration.

Table 2.1: Solutions for the isolation of protoplasts from *Vicia faba*

| **Enzyme Solution 1** | | **Enzyme Solution 2** | |
|---|---|---|---|
| 0.5 M | Mannitol | 0.5 M | Mannitol |
| 1 mM | $CaCl_2$ | 1 mM | $CaCl_2$ |
| 10 mM | Ascorbic acid | 10 mM | Ascorbic acid |
| 5mM | MES | 5mM | MES |
| 0.75% (w/v) | Cellulase | 1.5% (w/v) | Cellulase |
| 0.25% (w/v) | Macerozyme | 0.5% (w/v) | Macerozyme |
| | | 0.1% (w/v) | PVP25 |
| | | 0.1% (w/v) | BSA |
| | | 10 µg $ml^{-1}$ | Kanamycine |
| | | 10 µg $ml^{-1}$ | Cefotaxime |
| pH 5.7 (KOH) | | pH 5.7 (KOH) | |

| **Sucrose Solution** | | **Mannitol Solution** | |
|---|---|---|---|
| 0.6 M | Sucrose | 0.6 M | Mannitol |
| 1 mM | $CaCl_2$ | 1 mM | $MgCl_2$ |
| 10 mM | Ascorbic acid | 1 mM | $CaCl_2$ |
| 5mM | MES | 10 mM | Ascorbic acid |
| | | 5 mM | MOPS |
| pH 5.7 (KOH) | | pH 7.0 (NaOH) | |

### *2.2.2 Helianthus annuus*

To enable the use of protoplasts on the day of isolation and therefore avoid significant loss of viability, the incubation was carried out over night. Further two isolation procedures were compared which aimed to maintain bacterial numbers low:

1) Plant growth and protoplast isolation under sterile conditions;
2) Growth and isolation under non-sterile conditions including a digestion procedure with incubation of the leaf material in the fridge over night (4°C for 16 h).

All materials used during the isolation of sterile protoplasts were sterilised, either by autoclaving (Tuttnauer Systec ELV 3850, Systec GmbH, Wettenberg, Germany) (e.g. glassware, sucrose and salt solutions, see Table 2.2) or sterile filtration (enzyme solution, see Table 2.2). Approximately 2.0 g leaves were collected in a flow cabinet (HP 72, Gelaire

Flow Laboratories GmbH, Meckenheim, Germany) cut to pieces of about 25 $mm^2$ and floated upside down on 20 mL enzyme solution in a petri dish (diameter: 9.4 cm, height:1.6 cm). They were pre-incubated in the fridge for one hour and then transferred to a waterbath at 25°C. It was left to shake at low intensity (20 rpm) over night, before the temperature was increased to 28°C for two hours. Further steps were carried out as described below.

For the non-sterile protoplast isolation, the collection and treatment of the leaves were the same as for *V. faba*. The enzyme solution (2) is listed in Table 2.2. Following pre-incubation of ca. 4.0 g leaves in 40 mL enzyme solution in the fridge over night, the petri dish (diameter: 20 cm, height: 4.5 cm) was transferred into a waterbath at 28°C and shaken at low intensity (20 rpm).

Table 2.2: Solutions for the isolation of protoplasts from *Helianthus annuus*

| **Enzyme Solution 1** | | **Enzyme Solution 2** | | **Sucrose Solution** | | **Salt Solution** | |
|---|---|---|---|---|---|---|---|
| 0.1 % (w/v) | Cellulase | 1.0% (w/v) | Cellulase | 0.5 M | Sucrose | 335 mM | KCl |
| 0.02% (w/v) | Macerozyme | 0.5% (w/v) | Macerozyme | 1 mM | $CaCl_2$ | 13.6 mM | $CaCl_2$ |
| 0.05% (w/v) | Driselase | | | 5 mM | MES | 10 mM | MES |
| 1.0% (w/v) | BSA | 1.0% (w/v) | BSA | | | | |
| in salt solution pH 5.7 (KOH) | | in salt solution pH 5.7 (KOH) | | pH 5.7 (KOH) | | pH 5.7 (KOH) | |
| Schmitz, 1991 | | Schmitz, 1991 (modified) | | Müller, 2000 | | Müller, 2000 | |

After two hours, the enzyme solution containing protoplasts and cell fragments was passed through 200 µm and 50µm meshes and centrifuged (50 mL tubes, 100 g, 5 min, 20°C; Heraeus Minifuge GL). The supernatant was sucked off, the pellet resuspended in sucrose solution (Table 2.2), transferred to 10 mL test tubes and overlayed with 2 mL salt solution. Following centrifugation (20 g, 5 min, 20°C; Heraeus, Minifuge GL), the layer between the two solutions was sucked off. Any remaining sucrose solution was removed using salt solution (Table 2.2) during another centrifugation step (10 mL tubes, 100 g, 5min, 20°C; Heraeus Minifuge GL). The pellet was finally resuspended in a defined volume of salt solution. The protoplasts were counted using a Haemocytometer (Fuchs-Rosenthal) and stored in the fridge until further use.

## 2.3 Evacuolation

Protoplasts were evacuolated according to Griesbach and Sink (1983). A few modifications were necessary, since a fixed-angle rotor had to be used instead of a swing-out rotor. This enabled the evacuolation of a higher volume of the protoplast suspension. Emphasis had to be laid on the separation of the bands containing the desired mini-protoplasts (MPP) from the unwanted cell debris.

### *2.3.1 Vicia faba*

Different gradients were compared to evaluate their efficiency at separating the bands:

1) 100% (v/v) percoll + 0.5 M sorbitol, 100 mM $CaCl_2$ and 5 mM MOPS (pH: 7.0),
2) 100% (v/v) percoll buffer (Table 2.3),
3) Percoll buffer (Table 2.3) diluted 2:1 with 0.6 M mannitol solution (Table 2.1), and
4) Percoll buffer (Table 2.3) diluted 1:1 with 0.6 M mannitol solution (Table 2.1).

Of these solutions, 18 mL were filled into an ultracentrifuge tube (polycarbonate 26.3 mL; Beckman) and overlaid with 3 mL protoplast suspension. After centrifugation (119,000 g, 45 min, 23°C; Beckman Instruments Inc., Palo Alto, CA; Ultracentrifuge L7; SW 60 Ti rotor) the layer of mini-protoplasts (MPP) was sucked off, transferred into a 10 mL test tube containing 0.5 M mannitol solution (Table 2.1) and washed twice by centrifugation (150 g, 2 min, 20°C; Heraeus Minifuge GL). Finally, the pellet was resuspended in a defined volume of 0.5 M mannitol solution and stored in the fridge until further use.

Table 2.3: Percoll buffer for the evacuolation of *Vicia faba* and *Helianthus annuus* protoplasts

| *Percoll buffer* | | | |
|---|---|---|---|
| *Vicia faba* | | *Helianthus annuus* | |
| 100% (v/v) | Percoll | 100% (v/v) | Percoll |
| 600 mM | Mannitol | 600 mM | Sorbitol |
| 100 mM | $CaCl_2$ | 100 mM | $CaCl_2$ |
| 5 mM | MOPS | 5 mM | HEPES |
| pH 7.0 (MES / MOPS) | | pH 7.0 (MES / MOPS) | |

### *2.3.2 Helianthus annuus*

Similar to the case of *V. faba*, the gradient for the evacuolation of the *H. annuus* protoplasts had to be optimised to allow an adequate separation of the desired protoplasts from the debris. The following gradients and percoll solutions were tested:

1) Various dilutions of Percoll buffer:

   50% (v/v) Percoll buffer with 0.3 M sorbitol in salt solution (Table 2.2)

   50% (v/v) Percoll buffer with 0.3 M sorbitol in sucrose solution (Table 2.2)

   50% (v/v) Percoll buffer with 0.3 M mannitol in salt solution (Table 2.2)

   66% (v/v) Percoll buffer with 0.2 M sorbitol in sucrose solution (Table 2.2)

   50% (v/v) Percoll buffer with 0.3 M sorbitol in mannitol solution (Table 2.1)

   33% (v/v) Percoll buffer with 0.3 M sorbitol in salt solution (Table 2.2)

2) Discontinuous gradients of different percoll buffer concentrations diluted with salt solution (Table 2.2): three-step gradient: 90% (v/v), 60% (v/v) and 30% (v/v); two-step gradients 90% (v/v) and 60% (v/v); 80% (v/v) and 40% (v/v) percoll.

3) Percoll buffer diluted with salt solution (Table 2.2) to 70% (v/v), 80% (v/v) and 90% (v/v) percoll, pre-centrifuged (119,000 g, 25 min, 4°C; Beckman).

18 mL cooled percoll buffer (4°C) were filled into each ultracentrifuge tube (polycarbonate 26.3 mL; Beckman). Depending on the treatment, the percoll buffer was pre-centrifuged as described above, before overlaying it with 4 mL protoplast suspension. It was then centrifuged under the same conditions for 20 min. The layer of the mini-protoplasts was removed and washed twice with salt solution (Table 2.2) (3 5g, 5 min, 18°C; Heraeus Minifuge GL). The pellet was resuspended in a defined volume of salt solution (Table 2.2). The mini-protoplasts were counted using a Fuchs-Rosenthal Haemocytometer and stored in the fridge until further use.

## 2.4 Chlorophyll

The amount of chlorophyll in the protoplast suspension was determined after Arnon (1949) using a spectrophotometer (Kontron Uvikon 930, Kontron Instruments). 20 µL protoplast (PP) or mini-protoplast (MPP) suspension were added to 2 mL 80% acetone and centrifuged (3 min, 10,000 g; Mikroliter, Hettich Zentrifugen, Tuttingen). The extinction of the chlorophyll extracts (n=4) was measured at wavelengths of 645 nm and 663 nm against a reference of 80% acetone. The amount of chlorophyll was calculated as follows:

$$(E_{663} \times 8.02 + E_{645} \times 20.2) \times \text{total volume} / \text{sample volume} = \mu g \text{ chlorophyll} / \text{mL suspension}$$

## 2.5 Oxygen-Measurement

Oxygen production of PP and MPP was measured using a Clark electrode as a means of determining the viability of the suspension (Bornman and Bornman, 1985; Fitzsimons and Weyers, 1985). The electrode (928 6-Channel Oxygen System, Strathkelvin Instruments Ltd, Glasgow, UK) consisted of a platinum cathode and a silver anode, connected by a buffered electrolyte solution (Strathkelvin Instruments Ltd, Glasgow, UK) (Figure 2.1). The variation of each electrode was ±0.1% saturation or ±0.01 mg $O_2$ $L^{-1}$ (S. Davies, Strathkelvin Instruments Ltd, Glasgow, UK, personal communication, 1999).

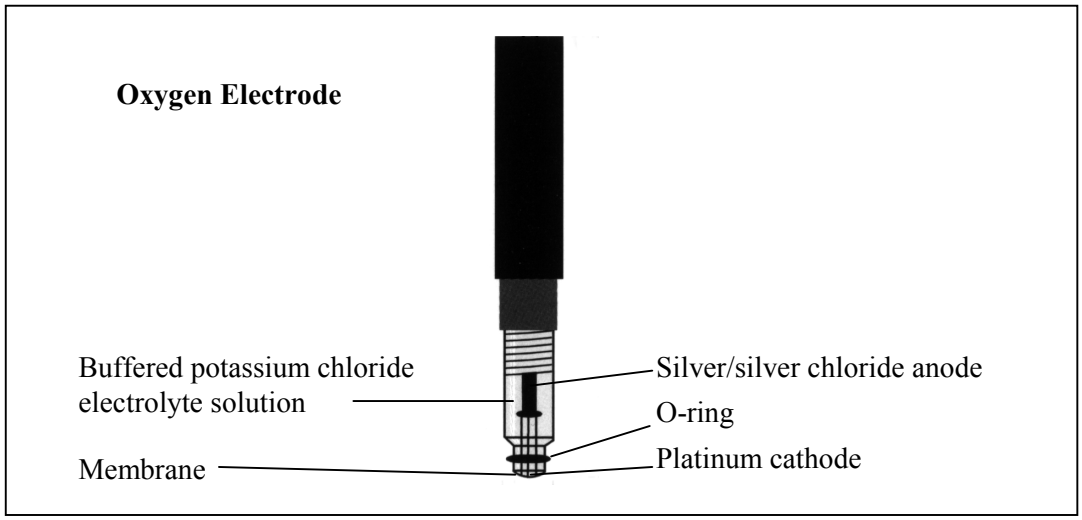



Figure 2.1: Diagrammatic presentation of a Clark-type oxygen electrode (Strathkelvin, 1999)

Each electrode was protected within an electrode holder, which prevented gaseous exchange between sample and atmospheric oxygen. A water jacket (Figure 2.2) allowed the maintenance of the sample temperature at 25°C by pumping temperated water around the measurement cell. A magnetic stirrer (Variomag Multipoint HP6, H+P Labortechnik GmbH, Oberschleißheim, Germany) maintained the protoplast suspension in motion, ensuring proper mixing of the oxygen in the water. A 150 Watt halogen lamp (Halolux 150, Streppel Glasfaser-Optik, Wermelskirchen, Germany) was set into each water jacket (2,5mm distance from measurement cell) (Figure 2.2) to illuminate the protoplast suspension for photosynthetic measurements (light intensity: 0.40 x $10^4$ $Em^{-2}$). For all measurements the water jackets with electrodes and samples were covered to shut out external light.

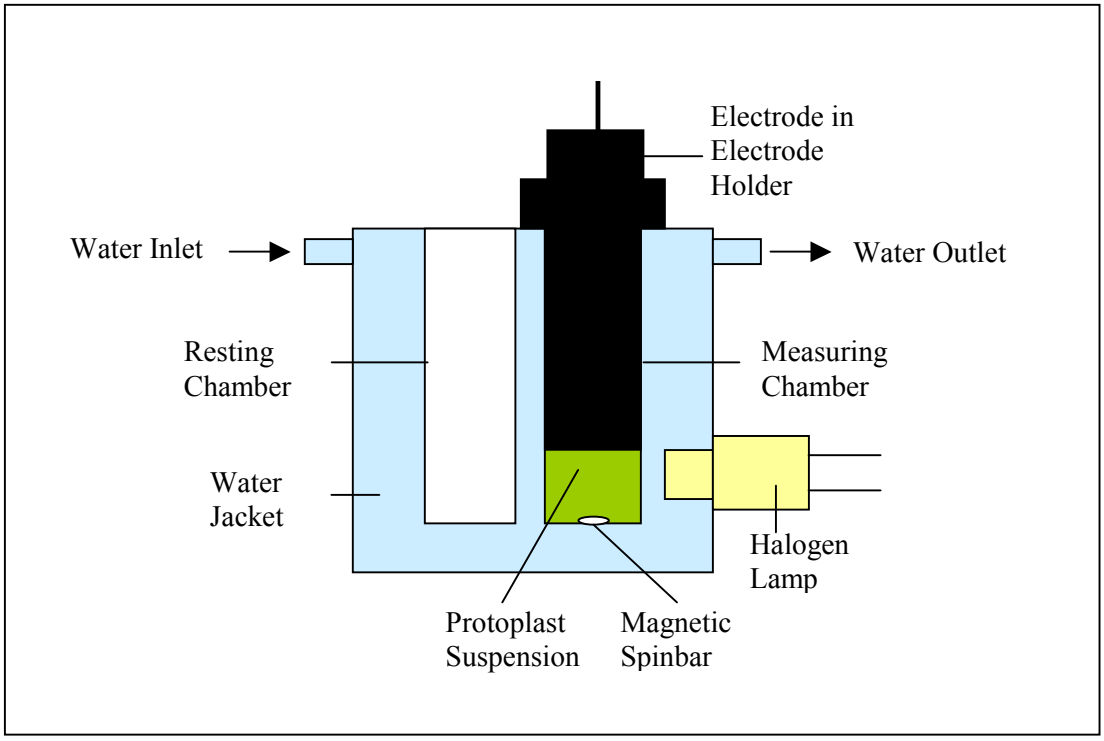



Figure 2.2: Water jacket holding a halogen lamp, a resting chamber and a measuring chamber (Strathkelvin Instruments Ltd, Glasgow, UK). During a measurement the electrode within the electrode holder was placed in the measuring chamber containing the protoplast or mini-protoplast suspension. A magnetic spinbar maintained the suspension in motion.

Figure 2.3 depicts all components of the oxygen measurement system. Temperated water was lead from the water bath into the water jackets on the magnetic stirrer. The six electrodes were connected to an interface (Interface 928 Oxygen System, Strathkelvin Instruments Ltd, Glasgow, UK). This provided the polarising voltage to the electrodes, amplified the low-level signals, carried out the A/D conversion, and send the data to the computer (Software: 928 Oxygen System, Strathkelvin Instruments). The halogen light source is responsible for the illumination of the protoplast suspension within the measurement chamber.

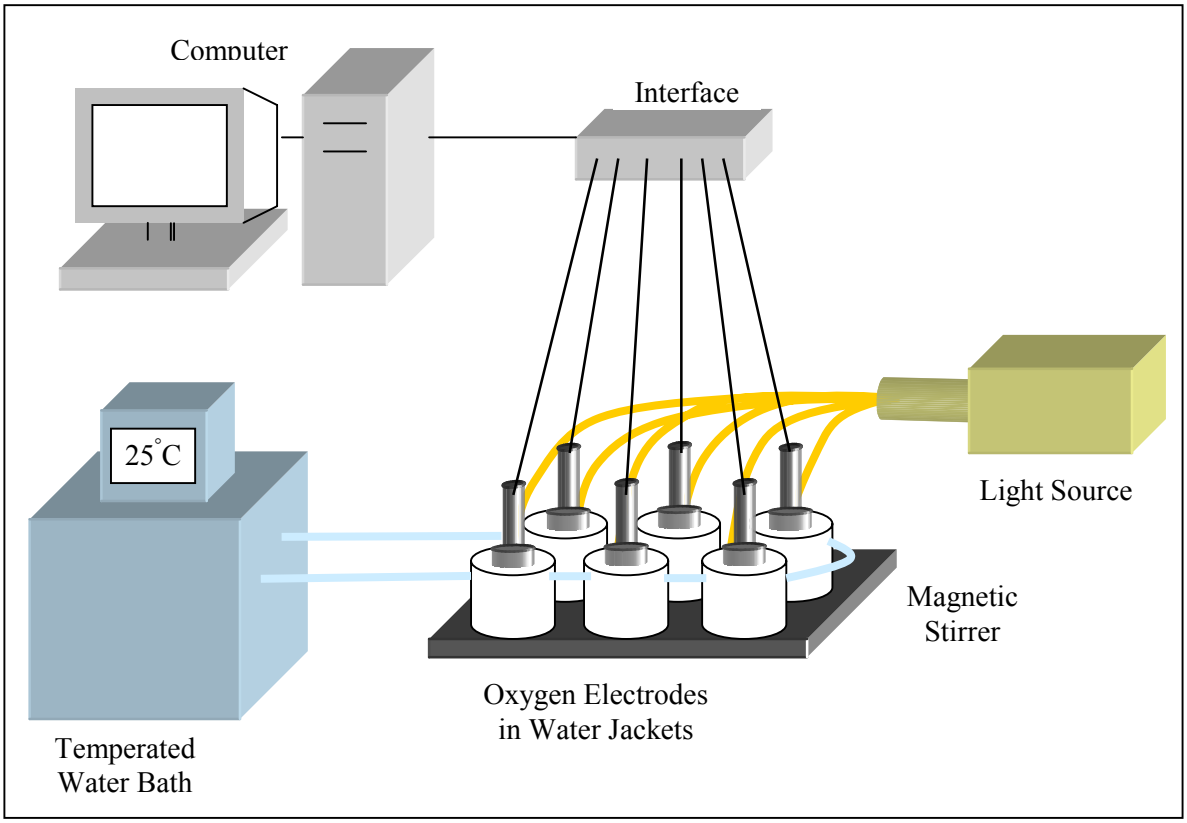



Figure 2.3: Oxygen measurement system: Six oxygen electrodes in the water jackets were standing on a magnetic stirrer. A water bath maintained the temperature of the sample at constant 25°C. A halogen lamp provided light for photosynthetic measurements. An interface sent the data from the electrodes to a computer.

To calibrate the system, 0% oxygen and air saturated distilled water were used. In water 0% oxygen was obtained by addition of sodium sulfite and 100% oxygen by bubbling air through the water. The calibration, as well as the measurements, were carried out at normal pressure and at a constant temperature of 25°C.

For each measurement a total volume of 1 mL was filled into the measurement cell, consisting of 400 µL measuring solution (Table 2.4), 20 µL $NaHCO_3$ (of 1 M stock solution), PP or MPP suspension with a chlorophyll content of 25 µg and made up to 1 mL with distilled water. Measurements were carried out over a 30 min period: 15 min in the dark, 15 min in the light. To determine the amount of oxygen consumed and produced, the gradient of the curve was determined over a 5 min period.

$$\frac{\text{Curve gradient}}{\text{Amount of chlorophyll or } 1 \times 10^5 \text{ PP or MPP}} = \text{µmol } O_2 \text{ / h / mg chlorophyll or } 1 \times 10^5 \text{ PP or MPP}$$

Table 2.4: Solution to measure oxygen-use and -evolution.

| **Measuring Solution** | |
|---|---|
| 2 M | Sucrose |
| 50 mM | HEPES |
| 10 mM | $MgCl_2$ |
| 5 mM | $CaCl_2$ |
| pH 8.2 (KOH) | |

## 2.6 Freezing

The protective properties were tested for four freezing media, namely 0.4 M trehalose (Treh 4), 0.6 M trehalose (Treh 6), 5% (v/v) glycerol (Glyc 5), 10% (v/v) glycerol (Glyc 10). Each medium contained 1 mM $CaCl_2$, 5% (w/v) BSA, 10 mM ascorbic acid, 0.1% (w/v) PVP, and 5 mM MES. PP or MPP suspensions containing about 150 µg chlorophyll were filled into Eppendorf caps and diluted to 1200 µL total volume using the freezing media. The suspensions were frozen at -20°C, the length of time depending on the individual experiments. It was thawed at 25°C and used for oxygen measurements, as described above.

## 2.7 Inhibitors

To determine the reactivity of the protoplasts towards substances which affect mitochondrial respiration, inhibitors were employed. Cyanide (KCN) was used to inhibit the cytochrome oxidase, and salicylhydroxamic acid (SHAM) to block the alternative oxidase. Stock solution of KCN and SHAM were made up in water and dimethyl sulfoxide, respectively, to concentrations of 100mM. After setting up a concentration curve, 1 mM KCN and 5 mM SHAM were used during respiration measurements. The percentage residual respiration was calculated by dividing the treated by the untreated protoplasts (controls). Non-acclimated and acclimated PP in different freezing media were compared with regard to their amount of oxygen-use and residual respiration when employing both inhibitors after the freeze-thaw cycle.

## 2.8 Fungicidal Substance

Fluazinam (analytical standard, 97.5% w/w purity; Zeneca), a pyridinamine fungicide, was added to the protoplast (acclimated, frozen in 10% (v/v) glycerol) suspension at different concentrations (10 nM, 100 nM, 1 µM, 10 µM, 100 µM, 1 mM, 10 mM) and the percentage residual respiration was determined. Oxygen measurements were also carried out with fluazinam at the indicated concentrations plus 1 mM KCN or/and 5 mM SHAM to evaluate the effect of the three inhibitors on oxygen-consumption.

## 2.9 Statistics

All statistical methods were carried out using SPSS 10.0 (SPSS Inc., 2000). To test for normal distribution, the Kolmogorov-Smirnov Test was used. Statistical differences were determined at a 5% confidence interval for all test.

First it was determined whether the oxygen measurements were influenced by methodological parameters or isolation factors. A univariate ANOVA was carried out to test if the individual electrodes or the time of the measurements affected the rates of oxygen-use or -evolution. The influence of the factors plant age, leaf number, cold-acclimation, and the amount of enzyme solution on the oxygen measurements were tested using bivariate correlations.

It was determined for *Vicia faba* whether the incubation time during the digestion procedure affected the protoplast yield, the amount of chlorophyll isolated per gram fresh weight leaf material, or the rates of oxygen-use and -evolution significantly using the univariate ANOVA. This test was also employed to see if the two antibiotic concentrations influenced the number of isolated protoplasts significantly and to detect the effects of the addition of tocopherol on oxygen-use and -evolution.

Furthermore, the test was used to determine the influence of sterile and non-sterile *H. annuus* protoplasts on the rates of oxygen-use and -evolution. To investigate whether protoplast yield and the amount of chlorophyll isolated of the two plant systems differed significantly, the Kolmogorov-Smirnov-Test was employed. Finally, the effect of plant age on protoplasts yield was evaluated using a univariate ANOVA.

The effect of methodological parameters during the evacuolation procedure was investigated by linear regression. It was determined whether the number of protoplasts per

mL used during evacuolation or the number of mL of protoplasts suspension added to each ultra-centrifuge tube significantly affected the mini-protoplast yield and their vitality.

To contrast the results of the protoplast isolations of *V. faba* and *H. annuus*, the amount of chlorophyll the Kolmogorov-Smirnov-Test was employed. The protoplast yields, the rates of oxygen-use and -evolution, and the percentage recovery after evacuolation were compared by univariate ANOVA.

The effects of cold-acclimation on the isolation of the protoplast yield and amount of chlorophyll were determined by univariate ANOVA. Similarly, the influence of the isolation parameters plant age, leaf number and amount of enzyme solution on protoplast yield and amount of chlorophyll isolated of NACC and ACC plants was tested. NACC and ACC, PP and MPP were compared with regard to their rates of oxygen-use and -evolution, using the univariate ANOVA.

This test was also used to detect significant differences of the effect of the four freezing media on the amount of oxygen-use. Univariate ANOVA was further employed to determine whether the addition of inhibitors (SHAM and/or KCN) had a significant effect on oxygen-use and percentage residual respiration of frozen protoplasts, and whether they influenced the inhibition of respiration by fluazinam.

# 3 RESULTS

The improvement of the biosensor, in view of its applicability under field conditions, involved three processes. First, an adequate donor plant had to be identified. The two plants *Vicia faba* and *Helianthus annuus* were compared with regard to protoplast yield and viability. Then a procedure needed to be developed to preserve the activity of the biological units over longer time periods. The most adequate procedure at this stage is freezing. Moreover, it was attempted to increase the mechanical stability of the protoplasts by removing the vacuole (evacuolation). Finally, the utilisation of the protoplasts was evaluated by determining their responsiveness towards respiration inhibitors and an agricultural fungicide was tested.

## 3.1 Validation of the Oxygen-Measurement System

As described in the introduction, the protoplast biosensor is based on oxygen-measurements. These involved the determination of the rates of oxygen-use of vital protoplasts in the dark and oxygen-evolution in the light: The measurements show a decrease in the amount of oxygen in the dark and an increase in the light, as is depicted by the typical curve in Figure 3.1. Any substances which interfere with respiration (oxygen-consumption) or photosynthesis (oxygen-evolution) will change the rates.

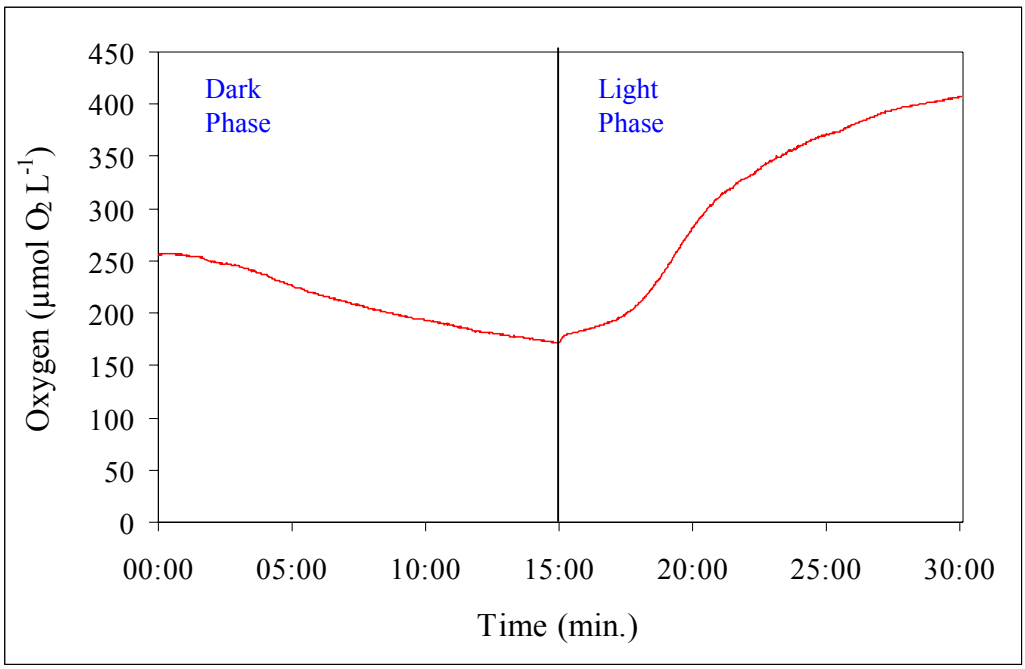



Figure 3.1: Example of an oxygen measurement curve: $O_2$-use in the dark and $O_2$-evolution in the light (dark and light phases over time periods of 15 minutes).

Since the oxygen-measurement is the parameter on which the biosensor is based, the influence of methodological factors on the rates of oxygen-use and -evolution had to be excluded. Several factors were investigated, including the electrodes, the age of the protoplasts, plant age, length of acclimation, leaf number, and amount of enzyme solution. The six individual electrodes used during the oxygen measurements did not influence the oxygen rates significantly. Similarly, the time sequence experiments showed no significant relationship between the age of the protoplasts and their consumption or production of oxygen. The coefficient of determination ($r^2$) indicates to which percentage one factor determines another. The bivariate correlation showed that the factors plant age, length of acclimation, leaf number, and amount of enzyme solution did not significantly affect oxygen-use nor oxygen-evolution of *Helianthus annuus* protoplasts (Table 3.1). This was also the case for the *Vicia faba* plants, with the exception of plant age, which correlated significantly with the oxygen measurements ($O_2$-use: $r^2$=0.913, n=6; $O_2$-evolution: $r^2$=0.691, n=6).

Table 3.1: Bivariate correlation between oxygen-use and -evolution of *Helianthus annuus* protoplasts and the isolation factors age of plants, length of acclimation, leaf number, and amount of enzyme solution (n=44).

| **Coefficient of Determination ($r^2$)** | **Oxygen-use** | **Oxygen-evolution** |
|---|---|---|
| Plant age | 0.004 | 0.002 |
| Acclimation | 0.003 | 0.003 |
| Enzyme solution | 0.001 | 0.044 |
| Leaf number | 0.043 | 0.005 |

## 3.2 Optimisation of Protoplast Isolation

To obtain an adequate biological unit for the protoplast biosensor, the two model plants *Vicia faba* and *Helianthus annuus* were compared as to their protoplast isolation efficiencies. First, the protoplast isolation procedures were optimised under consideration of the two criteria: protoplasts yield and vitality. The latter was determined through the use and evolution of oxygen by the protoplasts.

For *Vicia faba*, it was tested whether the already optimised, standard isolation procedure (4 h incubation) could be changed to include a 16 h incubation time during which the leaves remained in the enzyme solution. This was of importance, as storing the protoplasts (isolated using the 4 h incubation) in the fridge over night, resulted in a considerable loss of activity. Altering the procedure to include a slower digestion of cell wall materials

would enable protoplast isolation and oxygen measurements on the same day. Therefore, the standard isolation procedure (4 h incubation) and the new one (16 h) were compared. It was determined whether this new procedure could be optimised with regard to protoplast yield and activity. Tocopherol (vitamin E) was thus added to different stages of the *Vicia faba* protoplast isolation. To avoid bacterial damage of the protoplasts during the 16 h incubation at 25°C, antibiotics were added at different concentrations (10 µg $mL^{-1}$ or 50 µg $mL^{-1}$).

The protoplast isolation procedure for *Helianthus annuus* involved a 16 h incubation. Bacterial numbers were maintained low by: 1) sterile plant growth and protoplast isolation and 2) keeping the leaves in the enzyme solution in the fridge over night; therefore suppressing the need of antibiotics.

### *3.2.1 Isolation of Vicia faba Mesophyll Protoplasts*

It was determined whether the new procedures involving a 16 h incubation gave the same results as the standard procedure. The mean numbers of protoplasts per gram fresh weight (g f. wt.) leaf material isolated by the two procedures were not significantly different from each other. This was also the case for the amount of chlorophyll isolated per g f. wt.. The rate of oxygen-use was not influenced by incubation time. In contrast, the oxygen-evolution showed a significantly higher rate of 35.39 nmol $h^{-1}$ $\mu g^{-1}$ chlorophyll for the 4 h incubation and a lower rate of 18.69 nmol $h^{-1}$ $\mu g^{-1}$ chlorophyll for the 16 h incubation (Table 3.2).

Table 3.2: Descriptive statistics of incubation time of *Vicia faba* leaves in enzyme solution during protoplast isolation with regard to protoplasts yield, amount of chlorophyll isolated per gram fresh weight leaf material and the rates of oxygen-use and -evolution (SD: standard deviation; n: number of measurements; f. wt.: fresh weight; *nmol $h^{-1}$ $\mu g^{-1}$ chlorophyll; Sig: significance; NS: not significant (P>0.05); S: significant (P<0.05); test used: ANOVA).

| **Incubation time (h)** | **4 h** | | | **16 h** | | | **Sig.** |
|---|---|---|---|---|---|---|---|
| | **Mean** | **SD** | **n** | **Mean** | **SD** | **n** | |
| No PP (x $10^6$) $g^{-1}$ f. wt. | 0.91 | 0.15 | 3 | 1.29 | 0.32 | 8 | NS |
| µg Chlorophyll $g^{-1}$ f. wt. | 223.7 | 39.8 | 3 | 241.4 | 83.3 | 4 | NS |
| Oxygen-use * | 4.73 | 1.63 | 6 | 6.74 | 2.50 | 10 | NS |
| Oxygen-evolution * | 35.39 | 13.47 | 6 | 18.69 | 12.78 | 10 | S |

To maintain bacterial numbers low during the 16 h incubation of the leaf material at 25°C, the antibiotics kanamycin and cefotaxime were employed, both at concentrations of 10 µg

$mL^{-1}$ or 50 µg $mL^{-1}$. There was no significant influence of the antibiotic concentration on the protoplast yields (Table 3.3). Isolations without antibiotics were not included, as bacterial numbers were so large, that protoplasts were severely damaged. The lower concentration was used during further isolations.

Table 3.3: Descriptive statistics of the number of protoplasts isolated after the addition of the antibiotics kanamycin and cefotaxime to the digestion medium (SD: standard deviation; n: number; Sig: significance; NS: not significant (P>0.05); test used: ANOVA).

| **Kanamycin / Cefotaxime** | **Number of Protoplasts $g^{-1}$ fresh weight** | | | **Sig.** |
|---|---|---|---|---|
| | **Mean (x $10^6$)** | **SD** | **n** | |
| 10 µg $mL^{-1}$ | 1.14 | 1.07 | 4 | NS |
| 50 µg $mL^{-1}$ | 1.11 | 1.05 | 4 | NS |

Aiming at increasing the vitality of the isolated protoplasts tocopherol (vitamin E) was added to the protoplasts at different times (Chapter 2.2.1): to the digestion medium, after the clean-up procedure and before the removal of the vacuoles (evacuolation). Adding the tocopherol to the digestion medium did not influence the rates of oxygen-use or -evolution significantly at the tocopherol concentrations of 5 µM or 10 µM, compared to the control (Figure 3.2).

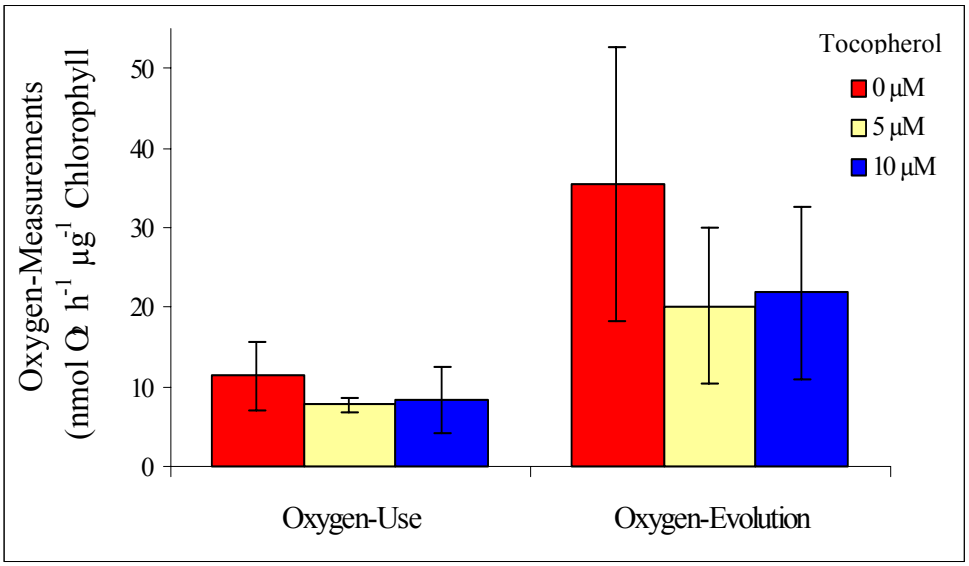



Figure 3.2: Comparison of the oxygen-use and -evolution (nmol $h^{-1}$ $\mu g^{-1}$ chlorophyll) of protoplasts isolated with 0, 5 or 10 µM tocopherol in the digestion medium.

It was further investigated, whether adding tocopherol to the protoplasts after the clean-up procedure would increase their oxygen-use and/or -evolution rates. Similarly to the

addition of the tocopherol to the digestion medium, no significant effects on oxygen-use, nor on oxygen-evolution, were detected (Figure 3.3).

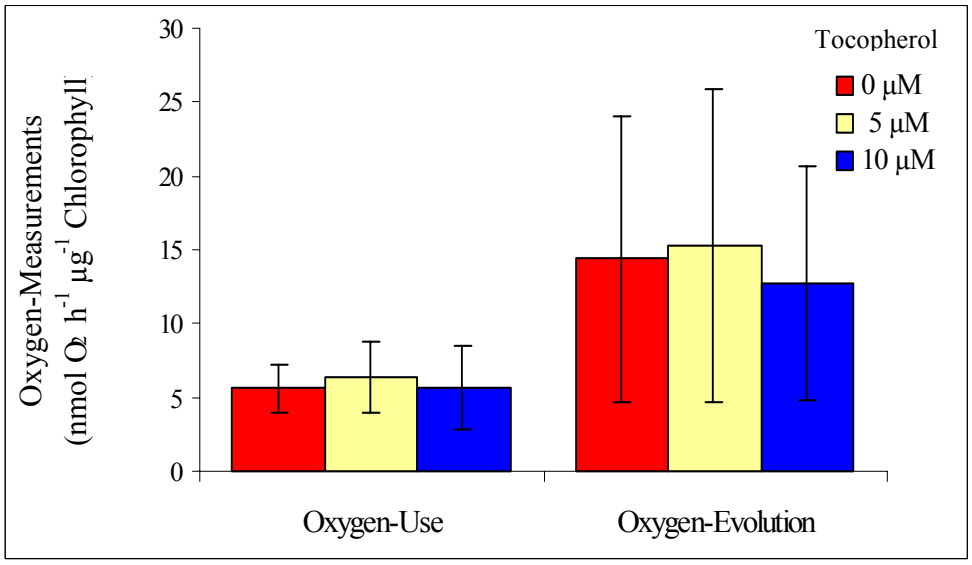



Figure 3.3: Comparison of the effects of different concentrations of tocopherol on oxygen-use and -evolution (nmol $h^{-1}$ $\mu g^{-1}$ chlorophyll) of protoplasts. Tocopherol (5 or 10 µM) was added to the protoplasts after the clean-up procedure.

Whether the addition of tocopherol to the protoplast suspension before evacuolation would increase the rates of oxygen-use and -evolution of the evacuolated protoplasts (mini-protoplasts; Chapter 2.3.1) was also investigated. This was not the case, since the two rates were not influenced significantly by the tocopherol (Figure 3.4).

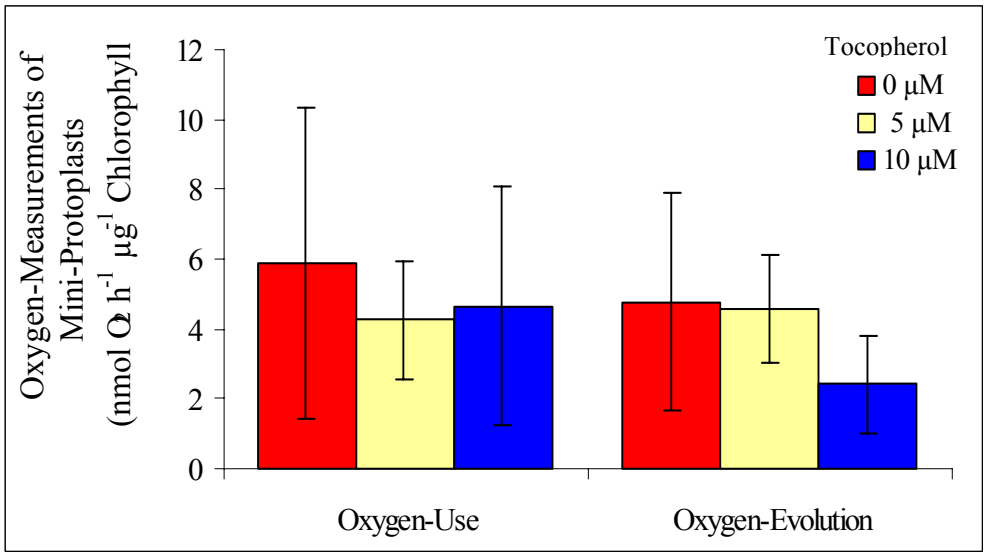



Figure 3.4: Comparison of the effects of different concentrations of tocopherol on oxygen-use and -evolution (nmol $h^{-1}$ $\mu g^{-1}$ chlorophyll) of mini-protoplasts. Tocopherol (5 or 10 µM) was added to the protoplasts before evaculoation.

### *3.2.2 Isolation of Helianthus annuus Mesophyll Protoplasts*

Since work on *H. annuus* regeneration in another research group of this Institute showed that the use of antibiotics during the isolation of protoplasts reduced their vitality (unpublished data), other options were tested to reduce bacterial numbers. Therefore, to avoid bacterial damage of the protoplasts, a sterile isolation was carried out, as well as a non-sterile one, in which the leaves on the enzyme solution were kept in the fridge over night.

*H. annuus* plants grown under sterile or non-sterile conditions showed phenotypic differences. The plants grown in Weck glasses were smaller. The leaves were also smaller, thinner and curlier than the those of the non-sterile plants. Differences were not only detected at the plant level but also at the protoplast level.

(a) (b)

Figure 3.5: (a) *H. annuus* plants grown under sterile conditions (Chapter 2.1.2) for three weeks. (b) *H. annuus* plants grown under non-sterile conditions (Chapter 2.1.1) for three weeks.

The protoplast yield, the amount of chlorophyll and the rates of oxygen-use and -evolution of the two isolation procedures (sterile and non-sterile) were compared. The mean yield of the sterile protoplasts was 3.72 x $10^6$ PP $g^{-1}$ f. wt., with 645.0 µg chlorophyll $g^{-1}$ f. wt.. In comparison, the protoplast yield of the non-sterile isolation was 6.82 x $10^6$ PP $g^{-1}$ f. wt. and the amount of chlorophyll 863.8 µg $g^{-1}$ f. wt.. These differences were significant for the protoplast yield and the amount of chlorophyll. The rates of oxygen-use and -evolution were also both significantly different between sterile and non-sterile protoplasts. The oxygen-use of the sterile isolation was 2.36 nmol $h^{-1}$ $\mu g^{-1}$ chlorophyll and of the non-sterile 5.51 nmol $h^{-1}$ $\mu g^{-1}$ chlorophyll. Similarly, the oxygen-evolution was lower for the

sterile: 7.13 nmol $h^{-1}$ $\mu g^{-1}$ chlorophyll than the non-sterile: 63.22 nmol $h^{-1}$ $\mu g^{-1}$ chlorophyll. Only the amount of chlorophyll of the protoplasts was not significantly different between the sterile (173.4 µg chlorophyll $(10^6$ PP$)^{-1}$ and the non-sterile (126.7 µg chlorophyll $(10^6$ PP$)^{-1}$ protoplasts (Table 3.4).

Table 3.4: Descriptive statistics of sterile and non-sterile *H. annuus* protoplasts with regard to protoplasts yield, amount of chlorophyll isolated per gram fresh weight leaf material, and the rates of oxygen-use and -evolution (SD: standard deviation; n: number of measurements; *nmol $h^{-1}$ $\mu g^{-1}$ chlorophyll; Sig: significance; NS: not significant (P>0.05); S: significant (P<0.05); test used: $O_2$ measurements: ANOVA, others: Kolmogorov-Smirnov-Test).

| | **Sterile** | | | **Non-sterile** | | | **Sig.** |
|---|---|---|---|---|---|---|---|
| | **Mean** | **SD** | **n** | **Mean** | **SD** | **n** | |
| No PP (x $10^6$) $g^{-1}$ f. wt. | 3.72 | 0.87 | 3 | 6.82 | 2.67 | 8 | S |
| µg Chlorophyll $g^{-1}$ f. wt. | 645.0 | 195.4 | 3 | 863.8 | 103.3 | 9 | S |
| µg Chlorophyll $(10^6$ PP$)^{-1}$ | 173.4 | 25.6 | 3 | 126.7 | 44.3 | 8 | NS |
| Oxygen-use * | 2.36 | 0.59 | 10 | 5.51 | 2.17 | 31 | S |
| Oxygen-evolution * | 7.13 | 1.88 | 9 | 63.22 | 29.55 | 31 | S |

To determine the effect of plant age on protoplast yield, the number of protoplasts isolated per gram fresh weight leaf material of two or three week old non-sterile plants was compared. The younger plants gave a mean number of 5.32 x $10^6$ PP $g^{-1}$ (SD= 2.00, n=4) and the older ones 4.97 x $10^6$ PP $g^{-1}$ (SD= 2.62, n=4), the difference not being significant. Thus, as an age difference of one week did not influence the protoplast yield.

### *3.2.3 Comparison between Vicia faba and Helianthus annuus*

The aim of comparing *Vicia faba* and *Helianthus annuus* was to determine the most appropriate plant to provide leaf protoplasts, as the biological unit for the biosensor, according to protoplast yield and activity. The protoplast yield of the non-sterile *H. annuus* was almost five times higher than for *V. faba*. This was also the case for the rate of oxygen-evolution. The rate of oxygen-use of the two protoplast types was not significantly different. Since very high numbers of biological units were needed to develop a procedure in order to preserve the activity of biological units through freezing, *H. annuus* was the more appropriate plant to isolate protoplasts from.

## 3.3 Evacuolation

The removal of the vacuole (evacuolation) has been reported to improve protoplast stability to handling (e.g. centrifugation, pipetting) (Burgess and Lawrence, 1985). Mesophyll protoplasts from both *Vicia faba* and *Helianthus annuus* were evacuolated, as the literature indicates enormous variations in the evacuolation potential of protoplasts from different plants. The existing evacuolation procedure (Griesbach and Sink, 1983) had to be modified for both protoplast tapes, since no band separation of mini-protoplasts or debris occurred using a fixed angle rotor (Chapter 2.3). After the development of a new procedure, the percentage recovery, oxygen-use and -evolution of the obtained mini-protoplasts (MPP) were determined.

### *3.3.1 Evacuolation of Vicia faba Mesophyll Protoplasts*

First of all, a procedure had to be developed to separate the desired MPP from the unwanted debris. Of the four different gradients (Chapter 2.3.1):

1) 100% percoll with 0.5 M sorbitol, $CaCl_2$ and MOPS,
2) 100% percoll buffer (100% percoll with 0.6 M mannital, $CaCl_2$ and MOPS),
3) Percoll buffer diluted 2:1 with 0.6 M mannitol solution, and
4) Percoll buffer diluted 1:1 with 0.6 M mannitol solution,

only the latter two lead to the band separation (Figure 3.6). Due to considerations possible harm of the percoll to the protoplasts, the 1:1 dilution of the percoll buffer was employed in further evacuolation procedures of *V. faba* protoplasts.

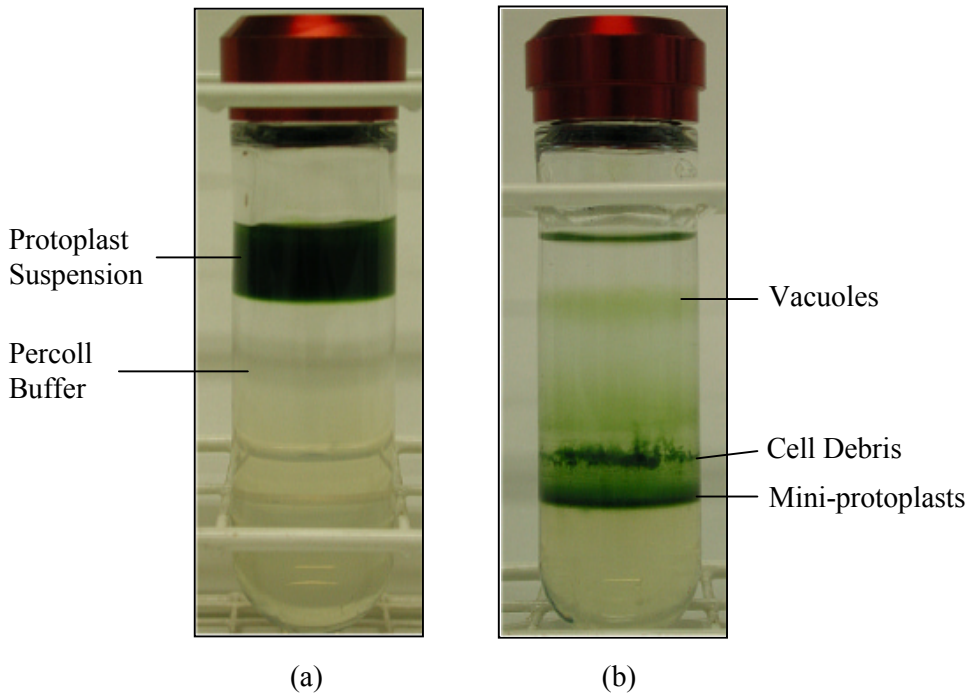



Figure 3.6: Band separation: (a) Percoll buffer overlaid with protoplast suspension before evacuolation. (b) Separated bands: vacuoles, cell debris and mini-protoplasts.

It was determined whether the methodology influenced the MPP yield or their vitality. The number of PP $mL^{-1}$ used during the evacuolation procedure did not influence the percentage recovery, oxygen-use or -evolution. These three indicators were also not affected significantly by the number of mL of PP-suspensions in each ultracentrifuge tube. The developed procedure lead to a recovery of MPP of 65.1% (SD: 19.5; n=5). The oxygen-use after evacuolation was 74.8% (SD: 16.6; n=6) and the oxygen-evolution 69.2% (SD: 26.6; n=6) of the non-evacuolated protoplasts (Figure 3.7). This shows that a remarkable number of protoplasts survived the removal of their vacuoles and maintained respiration and photosynthetic activities.

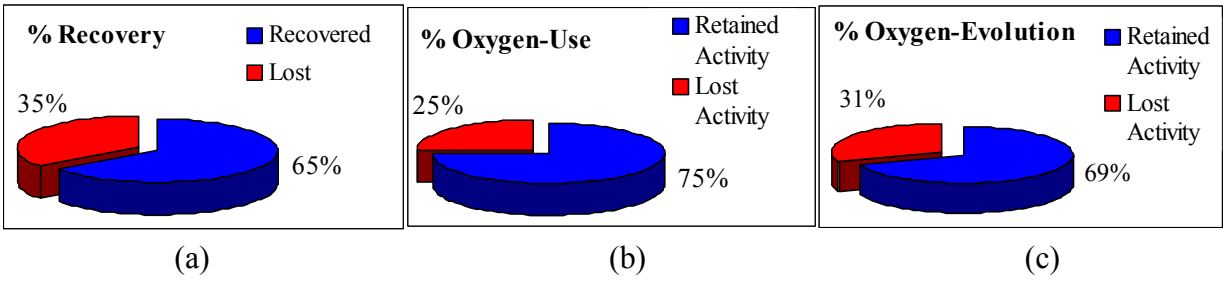
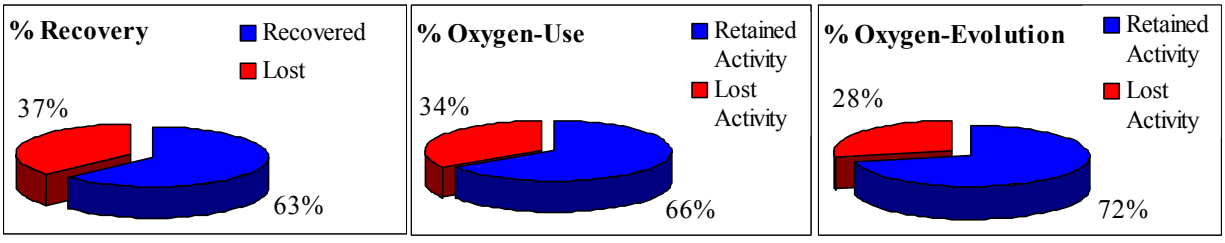



Figure 3.7: Percentage recovery of the number of *V. faba* mini-protoplasts (a) and their percentage oxygen-use (b) and -evolution (c) (levels before evacuolation =100%).

### *3.3.2 Evacuolation of Helianthus annuus Mesophyll Protoplasts*

Based on the successful separation of the mini-protoplasts from the debris during the evacuolation of the *V. faba* mesophyll protoplasts, a slightly adapted method for the *H. annuus* protoplasts was tried. Since no band separation was achieved, dilutions of the percoll buffer were carried out as described in chapter 2.3.2. However, no separation of the bands occurred. Therefore, different discontinuous gradients were employed, a three-step gradient at percoll buffer concentrations of 90% (v/v), 60% (v/v) and 30% (v/v), and two two-step gradients of 90% (v/v) and 60% (v/v) or 80% (v/v) and 40% (v/v). This did also not lead to a separation of the bands. The third set of trials again consisted of gradients using 70% (v/v), 80% (v/v), or 90% (v/v) percoll buffer in salt solution which were pre-centrifuged immediately before the evacuolation of the protoplasts (Chapter 2.3.2). These procedures resulted in the required separation, similar to the one depicted in Figure 3.6.

As for *V. faba*, the effect of the methodology of the evacuolation process on the percentage recovery, the oxygen-use and -evolution of *H. annuus* mini-protoplasts was investigated. These indicators were not significantly affected by the number of PP $mL^{-1}$ used for evacuolation, nor the number of mL of PP-suspension per ultracentrifuge tube. This evacuolation procedure resulted in a recovery of *H. annuus* mini-protoplasts of 63.0% (SD: 15.8; n=30) with rates of oxygen-use and -evolution of 66.1% (SD: 30.4; n=36) and 72.0% (SD: 19.8; n=36), respectively (Figure 3.8).




(a) (b) (c)

Figure 3.8: Percentage recovery of the number of *H. annuus* mini-protoplasts (a) and their percentage oxygen-use (b) and -evolution (c) (levels before evacuolation =100%).

### *3.3.3 Comparison between Vicia faba and Helianthus annuus*

The optimised method for the removal of the vacuoles of *V. faba* and *H. annuus* protoplasts lead to recoveries of 65% and 62%, respectively, the differences not being significant. The percentage recovery, oxygen-use and -evolution of the MPP were similar for both plants (Figure 3.7 and Figure 3.8). Due to the higher absolute numbers of *H. annuus*

MPP, as compared to *V. faba* MPP, the former were used to investigate the influence of cold-acclimation and evacuolation on the protoplast vitality - an experiment which has not been described in the literature up to now.

Since, however, relatively low numbers of protoplasts could be evacuolated at the laboratory scale and increasing the MPP yield from this small to a large-scale, industrial production was beyond the scope of this project, normal protoplasts were used for further investigations. These included the development of a freezing procedure and the determination of the responsiveness of the unfrozen and frozen protoplasts to respiration inhibitors. Transferring the obtained results of the investigations to MPP and increasing the production in scale can set the basis for a future project.

In conclusion, the most adequate unit to develop a procedure to preserve the activity of the biological component of the biosensor, were the non-sterile *H. annuus* protoplasts with vacuoles. This was due to the significantly higher yield of *H. annuus* mesophyll protoplasts compared to the *V. faba*, as large numbers of protoplasts were needed for the oxygen measurements.

## 3.4 Protection of Protoplast Activity

In order to get biological units for the biosensor, which are available at any time, protoplast activity should be preserved over longer time periods. To protect the protoplasts from obtaining lethal injuries during the freeze/thaw cycle, the effect of cold-acclimation and cryoprotectants on protoplast activity was determined.

### *3.4.1 Cold-Acclimation*

To increase the tolerance of the cells to freezing even before tissue digestion, *Helianthus annuus* plants were acclimated to cold conditions (8°C). The acclimation process (Chapter 2.1.1) lead to phenotypical changes in the leaves, being more yellow (Figure 3.9b) compared to those of plants grown at higher temperatures (Figure 3.9a).

(a) (b)

Figure 3.9: (a) Leaf of three week old, non-acclimated *Helianthus annuus* plant. (b) Leaf of six week old, cold-acclimated *Helianthus annuus* plant.

Significant differences were detected between the yield, vitality and amount of chlorophyll of the protoplasts from non-acclimated (NACC) and acclimated (ACC) plants. The protoplast yield of the NACC leaves was significantly higher (5.87 x $10^6$ PP $g^{-1}$ f. wt.) than of the ACC leaves (4.25 x $10^6$ PP $g^{-1}$ f. wt.) (Table 3.5). The mean amount of chlorophyll per g f. wt. was also significantly greater in the former (776.5 µg chlorophyll $g^{-1}$ f. wt.) than the latter (357.3 µg chlorophyll $g^{-1}$ f. wt.) (Table 3.5). These difference were visible in the protoplasts (Figure 3.10), the NACC showing more chloroplasts than the ACC ones.

Table 3.5: Descriptive statistics of the number of protoplasts and the amount of chlorophyll isolated per g f. wt. leaf material of non-acclimated (NACC) and acclimated (ACC) *H. annuus* plants (SD: standard deviation; n: number of experiments; Sig: significance; S: significant (P<0.05); test used: ANOVA).

| | **NACC** | | | **ACC** | | | **Sig.** |
|---|---|---|---|---|---|---|---|
| | **Mean** | **SD** | **n** | **Mean** | **SD** | **n** | |
| No PP (x $10^6$) $g^{-1}$ | 5.87 | 2.79 | 45 | 4.25 | 2.81 | 16 | S |
| µg Chlorophyll $g^{-1}$ | 776.5 | 279.2 | 29 | 357.3 | 210.1 | 12 | S |

In order to disregard isolation parameters as a reason for these differences, it was determined whether the protoplast yields or amount of chlorophyll of the two differently treated plants were influenced by the parameters plant age, leaf number and amount of enzyme solution used during digestion. The number of protoplasts isolated per g NACC leaves was not significantly influenced by any of the three factors, as was the case with

ACC protoplasts. Similarly, the amount of chlorophyll per g f. wt. leaf material was not influenced significantly by the three factors, neither with NACC, nor with ACC leaves. Therefore, the variations in protoplast yield and chlorophyll were not due to these methodological parameters.

It was further investigated whether cold-acclimation influenced the rates of oxygen-use and -evolution (Table 3.6). The rates of oxygen-consumption were 145.53 nmol $h^{-1}$ $10^5$ $PP^{-1}$ for the NACC protoplasts and 142.87 nmol $h^{-1}$ $10^5$ $PP^{-1}$ for the ACC ones. The NACC protoplasts had a rate of oxygen-evolution of 31.70 nmol $h^{-1}$ $\mu g^{-1}$ chlorophyll and the ACC protoplasts showed a rate of 26.79 nmol $h^{-1}$ $\mu g^{-1}$ chlorophyll. No significant differences between acclimated and non-acclimated protoplasts were found with regard to both their rates of oxygen- use and -evolution.

#### *3.4.1.1 Cold-Acclimation and Evacuolation*

The effects of cold-acclimation were determined with regard to the evacuolation of protoplasts. Looking at NACC and ACC MPP showed that the rates of oxygen-use and -evolution varied significantly. The removal of the vacuoles lead to lower rates of oxygen-use for both, NACC MPP and ACC MPP compared to their respective protoplasts. The ACC MPP, however, showed an even lower oxygen-consumption than the NACC MPP. Significantly lower rates of oxygen-evolution were detected for NACC MPP, compared to NACC non-evacuolated protoplasts. This was not the case for the ACC MPP, which revealed the same rates of oxygen production as their respective normal protoplasts (Table 3.6). In conclusion, the cold-acclimation of *H. annuus* plants seems to have affected the protoplasts, leading to differences in their tolerance of the evacuolation procedure. The removal of the vacuoles lead to less oxygen-consumption of ACC MPP than NACC MPP but higher oxygen-evolution for former than the latter. Therefore, while acclimation lead to preservation of oxygen-evolution during evacuolation of protoplasts, a greater loss of oxygen-uptake than for NACC protoplasts was revealed.

Table 3.6: Descriptive statistics of oxygen-use and -evolution of non-acclimated (NACC) and acclimated (ACC) protoplasts (PP) and mini-protoplasts (MPP) of *H. annuus* leaves in salt solution (SD: standard deviation; n: number of measurements; [a-e]: statistical differences (ANOVA): same letters: no statistical difference (P>0.05), different letters: difference statistically significant (P<0.05)).

| **$O_2$-Measurement** | **NACC PP** | | **ACC PP** | | **NACC MPP** | | **ACC MPP** | |
|---|---|---|---|---|---|---|---|---|
| | **Mean (SD)** | **n** | **Mean (SD)** | **n** | **Mean (SD)** | **n** | **Mean (SD)** | **n** |
| $O_2$-Use (nmol $h^{-1}$ $10^5$ $PP^{-1}$) | 145.53[a] (92.48) | 75 | 142.87[a] (78.77) | 89 | 50.09[b] (15.76) | 19 | 22.59[c] (8.20) | 18 |
| $O_2$-Evolution (nmol $h^{-1}$ $\mu g^{-1}$ chlorophyll) | 31.70[d] (22.20) | 71 | 26.79[d] (22.11) | 84 | 11.69[e] (6.59) | 21 | 28.18[d] (13.72) | 18 |

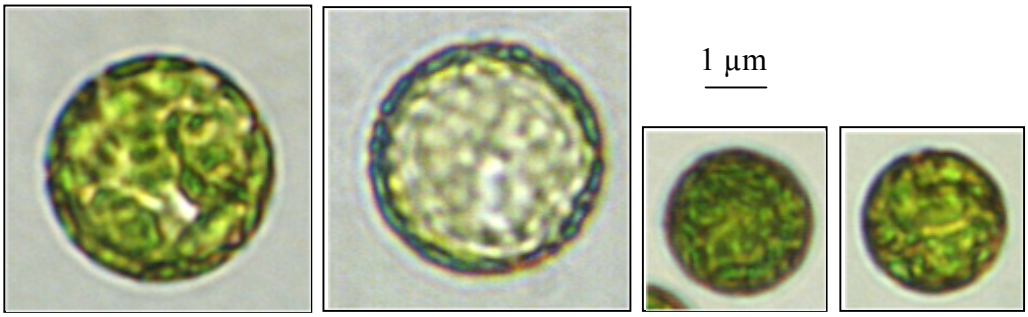



Figure 3.10: Examples of non-acclimated (NACC) or acclimated (ACC) protoplasts (PP) or mini-protoplasts (MPP). Cold acclimation of plants at 8°C.

### *3.4.2 Freezing Helianthus annuus Mesophyll Protoplasts*

Protoplasts were frozen to preserve their activity over longer time periods. To protect them more effectively from injury during the freeze/thaw cycle, they were frozen in four different media: 0.4 M trehalose (Treh 4), 0.6 M trehalose (Treh 6), 5% (v/v) glycerol (Glyc 5), and 10% (v/v) glycerol (Glyc 10) (Chapter 2.6). The effect of cold-acclimation on the freezing survival was investigated by comparing the rates of oxygen-use and -evolution of NACC and ACC protoplasts after the completion of a freeze/thaw cycle.

The four media clearly affected the rate of oxygen-evolution, as did cold-acclimation (Figure 3.11). This became evident after freezing the NACC PP for 30 minutes: only those frozen in glycerol solutions maintained oxygen-production. In comparison, the ACC PP showed oxygen-evolution after 30 minutes in all four media. After 45 minutes, no oxygen-evolution could be detected for NACC PP, while it was detectable for ACC PP frozen in Glyc 10. This showed that both procedures, acclimation and cryoprotection, affected the rate of oxygen-production.

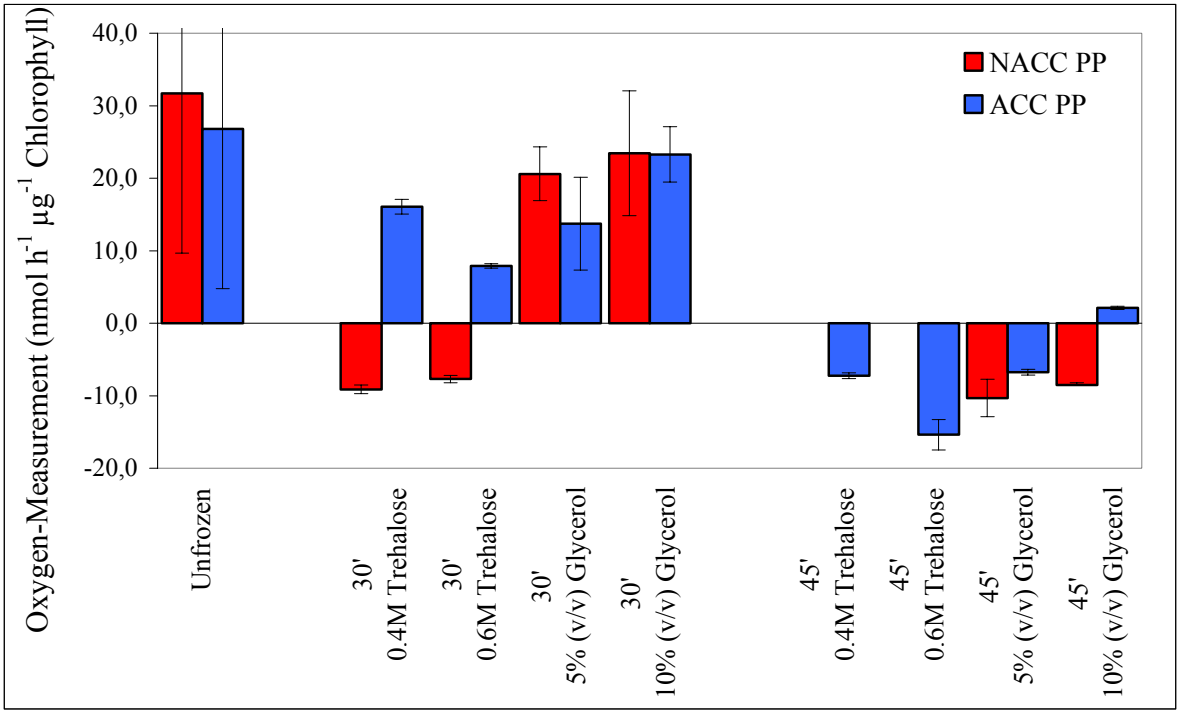



Figure 3.11: Oxygen-evolution of non-acclimated (NACC) and acclimated (ACC) protoplasts (PP) in different freezing media.

In contrast, no significant influence of the four media was measured on the rates of oxygen-use of both protoplast types (NACC and ACC) (Figure 3.12). The rates of oxygen-use of the NACC PP in Treh 4, Treh 6, Glyc 5, and Glyc 10 were 198.01, 197.18, 172.73, and 168.84 nmol $h^{-1}$ $10^5$ $PP^{-1}$, respectively. The ACC PP showed rates of oxygen-consumption of 126.22, 130.85, 136.24, and 142.39 nmol $h^{-1}$ $10^5$ $PP^{-1}$ in Treh 4, Treh 6, Glyc 5, and Glyc 10, respectively. As can be seen, for all four media the rates of oxygen-use of ACC PP were significantly lower than those of the NACC ones. Only the unfrozen controls (protoplasts in salt solution) showed no significant differences in the oxygen rates of the two protoplast types. Therefore, the only significant difference was found in the rates of oxygen-use of NACC and ACC PP after the completion of a freeze/thaw cycle, while the media showed no significant effect.

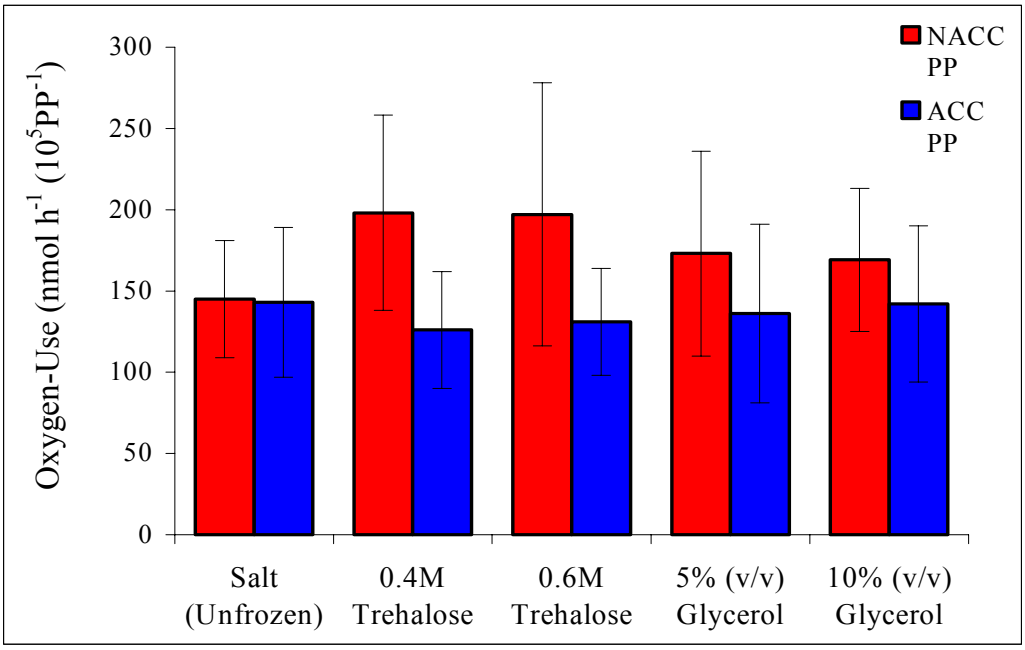



Figure 3.12: Oxygen-use of non-acclimated (NACC) and acclimated (ACC) protoplasts (PP); protoplasts in salt solution: unfrozen; in trehalose and glycerol solutions: measurements ($n \geq 42$) were carried out after completion of the freeze/thaw cycle.

It was further investigated whether freezing protoplasts over a longer time period affected the rate of oxygen-uptake. Protoplasts of NACC and ACC plants were frozen in Glyc 10 solution (as it showed to provide most protection) for one hour, one day or three months and their rates of oxygen-use were compared. After three months of freezing, both protoplasts types showed a reduced oxygen-uptake compared to those frozen for shorter time periods (Figure 3.13). This reduction was less for ACC than NACC protoplasts. While NACC PP frozen for three months maintained 61% oxygen-consumption, the ACC PP kept 70% oxygen-uptake after the three months, compared to the unfrozen controls. Thus, a decrease in the rate of oxygen-consumption occurred when freezing the protoplasts for a longer time period, the reduction being less for ACC than NACC protoplasts.

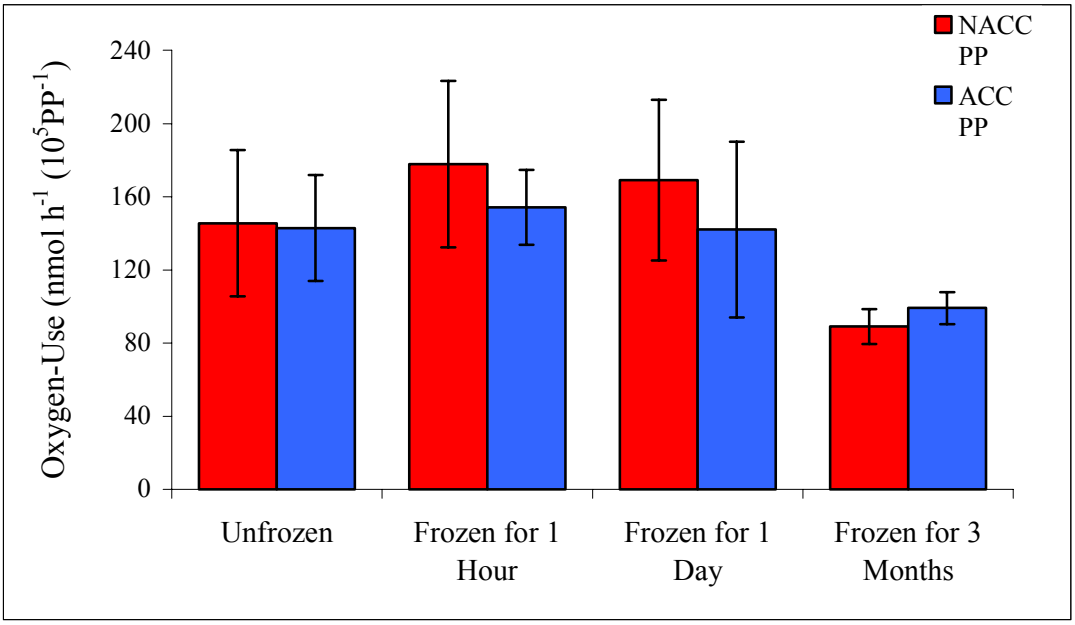



Figure 3.13: Oxygen-use of non-acclimated (NACC) and acclimated (ACC) protoplasts (PP) in 10% (v/v) glycerol solution after one hour or three months freezing.

## 3.5 Responsiveness of the Biological Units

Inhibitors of mitochondrial respiration were employed to determine the responsiveness of the non-acclimated and acclimated protoplasts towards these substances. Since plant mitochondria have two pathways for electron transfer to oxygen (Chapter 1.2) two inhibitors had to be used to block both: KCN was employed to inhibit the cytochrome pathway and SHAM to block the alternative pathway.

### *3.5.1 Effects of Respiration Inhibition on Protoplasts*

Treating protoplasts with the two inhibitors, singly or in combination, resulted in the oxygen measurement curves depicted in Figure 3.14. These three curves and the untreated control all differed in their appearances: the untreated control showed a steady use of $O_2$ over time (Figure 3.14) eventually resulting in the complete consumption of the oxygen (not shown). The addition of SHAM to the protoplasts resulted in a reduced rate of oxygen-use without changing the shape of the curve. Treating the protoplasts with KCN, on the other hand, lead to an increased oxygen-use within the first minute before the rate was reduced. The measurement with KCN + SHAM was similar to that with KCN only, except that the slope of the former curve was steeper within the first two minutes, before levelling off (Figure 3.14). Thus, the treatment of the protoplasts with the two inhibitors clearly affected the rate of oxygen-consumption.

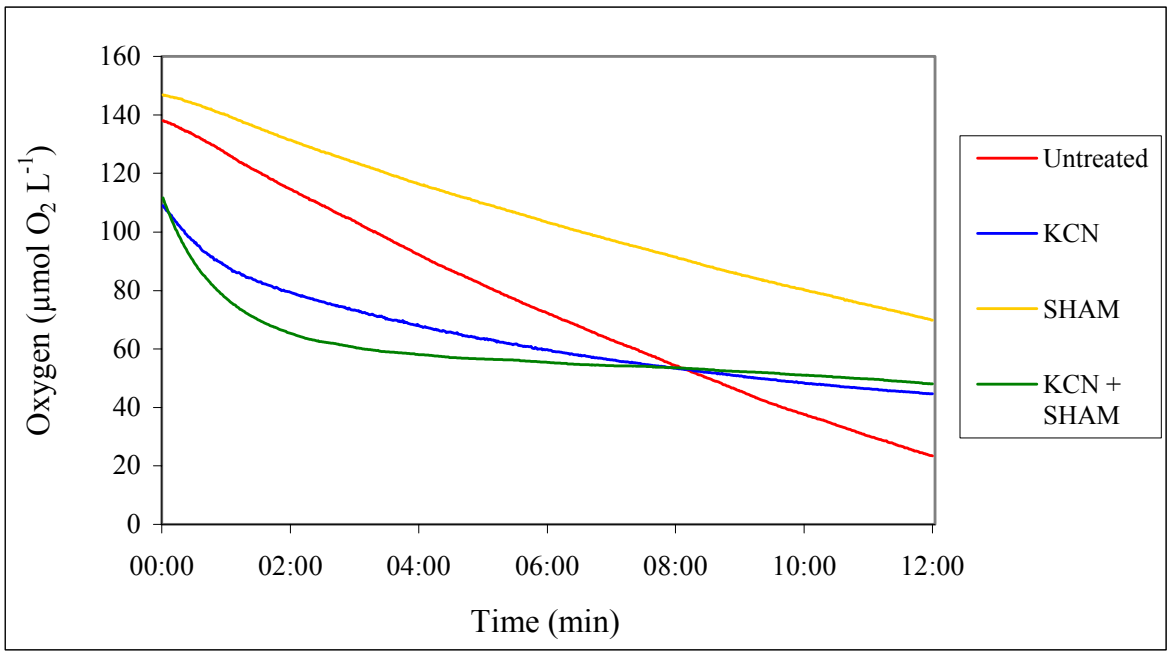



Figure 3.14: Examples of oxygen measurements of untreated (control) and treated *Helianthus annuus* protoplasts. Treatment consisted of the addition of cyanide (KCN, 1 mM) and salicylhydroxamic acid (SHAM, 5 mM), singly or in combination, to the protoplast suspension.

The treatment of the protoplasts with KCN and/or SHAM influenced the rate of oxygen-use and the residual respiration (percentage remaining oxygen-consumption of untreated control) significantly. These two indicators (oxygen-use and residual respiration) were highest for NACC and ACC protoplasts when applying SHAM, as compared to KCN or KCN + SHAM. The latter two treatments were not significantly different from each other. Nor did the oxygen-use and residual respiration of the two protoplast types vary significantly (Table 3.7).

Table 3.7: Descriptive statistics of oxygen-use and residual respiration when employing the respiration inhibitors SHAM (5 mM) or/and KCN (1 mM) on non-acclimated (NACC) and acclimated (ACC) protoplasts (PP) in salt solution (SD: standard deviation; n: number; [a-h]: statistical differences (ANOVA): same letters: no statistical difference (P>0.05), different letters: difference statistically significant (P<0.05)).

| Protoplasts | Treatment | Oxygen-Use (nmol $h^{-1}$ $10^5$ $PP^{-1}$) | | | % Residual Respiration | | |
|---|---|---|---|---|---|---|---|
| | | Mean | SD | n | Mean | SD | n |
| NACC | KCN | 71.37[a] | 22.85 | 24 | 51.1[c] | 16.3 | 24 |
| | SHAM | 118.12[b] | 33.16 | 24 | 84.1[d] | 16.8 | 23 |
| | KCN + SHAM | 68.74[a] | 23.70 | 24 | 48.9[c] | 18.3 | 23 |
| ACC | KCN | 57.71[e] | 28.32 | 23 | 41.3[g] | 16.2 | 22 |
| | SHAM | 101.67[f] | 38.28 | 17 | 72.8[h] | 25.4 | 16 |
| | KCN + SHAM | 40.96[e] | 20.67 | 17 | 29.3[g] | 12.6 | 16 |

To set a basis for transferring the results on the freezing procedure developed using protoplasts onto MPP, first investigations were carried out to determine the responsiveness of the MPP towards respiration inhibitors. ACC MPP revealed a residual respiration of 27.4% after the addition of KCN and SHAM. This value was similar to the one measured for ACC protoplasts (Table 3.7).

### *3.5.2 Response of Frozen Protoplasts towards Inhibitors*

When adding both inhibitors to NACC and ACC protoplasts, the rate of oxygen-use varied significantly according to the medium in which the protoplasts were frozen. For NACC PP the highest rates of oxygen-use were found in Treh 6 (113.78 nmol $h^{-1}$ $10^5$ $PP^{-1}$) and Treh 4 (113.06 nmol $h^{-1}$ $10^5$ $PP^{-1}$). The protoplasts frozen in the Glyc 10 gave a significantly lower rate (60.04 nmol $h^{-1}$ $10^5$ $PP^{-1}$) than in trehalose solutions. The Glyc 5 medium had a rate of 84.35 nmol $h^{-1}$ $10^5$ $PP^{-1}$). The unfrozen controls in salt solution showed the lowest values (39.44 nmol $h^{-1}$ $10^5$ $PP^{-1}$), being significantly different from the other measurements (Figure 3.15).

The results of adding KCN + SHAM to ACC PP in the various media were very similar to those described for NACC PP. The oxygen-use was highest for protoplasts in the two trehalose solutions (Treh 6: 77.39 nmol $h^{-1}$ $10^5$ $PP^{-1}$, Treh 4: 75.73 nmol $h^{-1}$ $10^5$ $PP^{-1}$) and the Glyc 5 (57.91 nmol $h^{-1}$ $10^5$ $PP^{-1}$). Those in Glyc 10 had the lowest levels (38.32 nmol $h^{-1}$ $10^5$ $PP^{-1}$) of the four media. No significant difference was found between the protoplasts frozen in Glyc 10 medium and the unfrozen controls in salt solution (46.08 nmol $h^{-1}$

$10^5$ $PP^{-1}$) (Figure 3.15). This latter finding stood in contrast to the results obtained with the NACC PP. Thus, the only biological units which, showed no difference in oxygen-use between the unfrozen control and having completed the freeze/thaw cycle were the ACC PP in Glyc 10 solution.

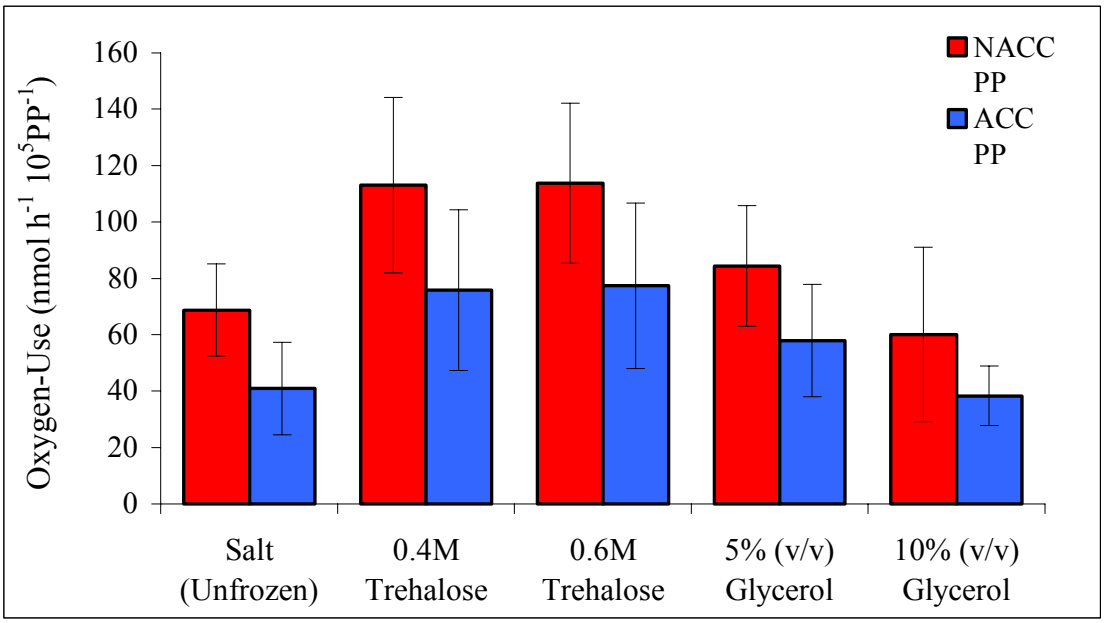



Figure 3.15: Oxygen-use of non-acclimated (NACC) and acclimated (ACC) protoplasts (PP) in different media after treatment with 1 mM KCN and 5 mM SHAM solution; protoplasts in salt solution: unfrozen; in trehalose and glycerol solutions: measurements ($n \geq 40$) were carried out after completion of the freeze/thaw cycle.

The percentage oxygen-use of both ACC and NACC protoplasts emphasises the effect of the freezing media on the inhibition of respiration. The same pattern could be seen, as depicted in Figure 3.14, where the trehalose and the glycerol solutions, as well as the trehalose and the salt solution showed significant difference in oxygen-uptake. The lowest percentage inhibition of respiration and thus greatest residual respiration was measured for protoplasts frozen in the trehalose media. The greatest inhibition of respiration, on the other hand, was found for the unfrozen control protoplasts, as well as the ACC PP frozen in Glyc 10 solution. Both had statistically the same values. Thus, it was possible to reduce the oxygen-use of the protoplasts in Glyc 10 solution by about 70%, while those in trehalose medium only had a reduction of around 40%, even when the mitochondrial electron transport was entirely blocked. Due to this greater possible variation in oxygen-uptake of protoplasts frozen in the Glyc 10 solution, as well as the higher similarity between them and the unfrozen control, this freezing medium was used to evaluate the effect of freezing the protoplasts for three months.

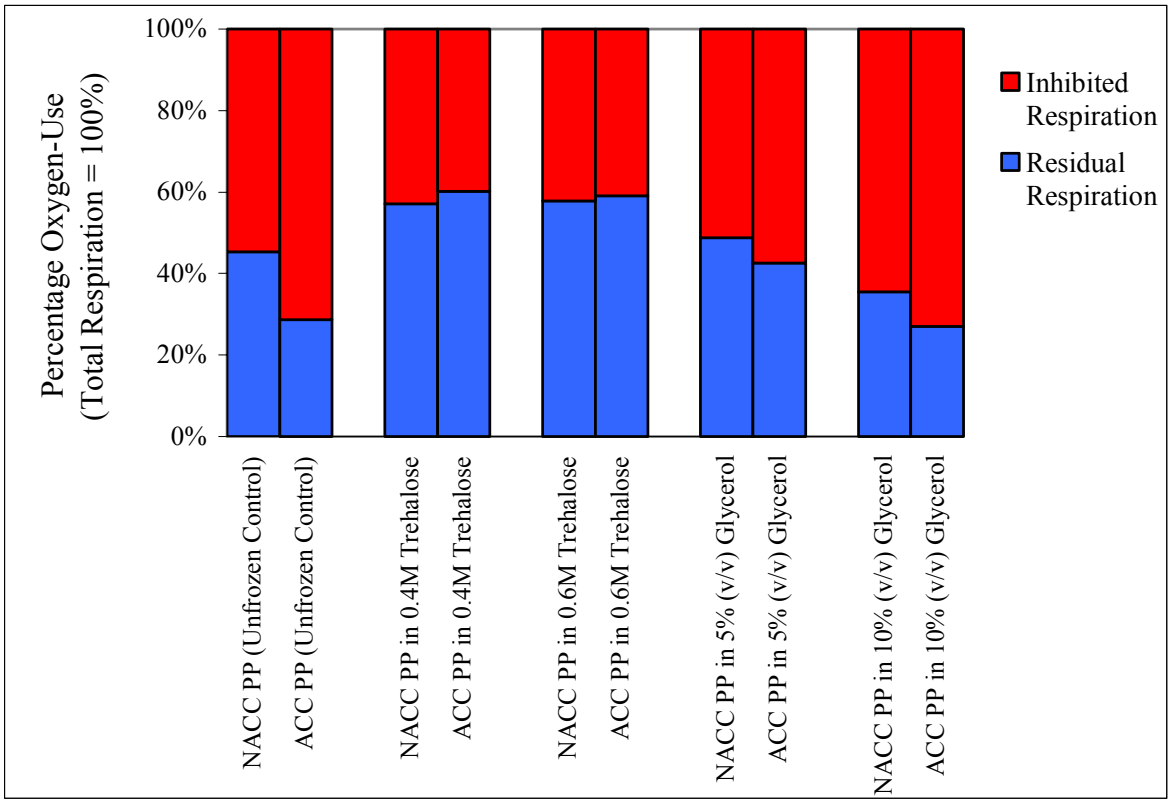



Figure 3.16: Percentage oxygen-use of non-acclimated (NACC) and acclimated (ACC) protoplasts (PP) in different media after treatment with 1 mM KCN and 5 mM SHAM. Total respiration (= 100%) consisted of inhibited and residual respiration. Inhibited respiration: amount of oxygen-uptake inhibited by protoplast treatment with respiration inhibitors; Residual respiration: amount of oxygen-uptake not inhibited by protoplast treatment with respiration inhibitors; Protoplasts in salt solution: unfrozen; In trehalose and glycerol solutions: measurements ($n \geq 40$) were carried out after completion of the freeze/thaw cycle.

The responsiveness of the protoplasts frozen for three months towards respiration inhibitors was also determined. Both protoplast types showed a lower percentage inhibition of respiration after freezing for three months compared to the unfrozen controls or those frozen for one day (Figure 3.17). Greater inhibition of respiration (i.e. less residual respiration) was apparent for ACC than NACC protoplasts, regardless of whether they were frozen or not. Therefore, while after three months of freezing the ACC protoplasts showed a higher rate of oxygen-use than the NACC protoplasts, the percentage residual respiration of the former was lower than of the latter. Consequently, the ACC protoplasts were employed for the following experiment.

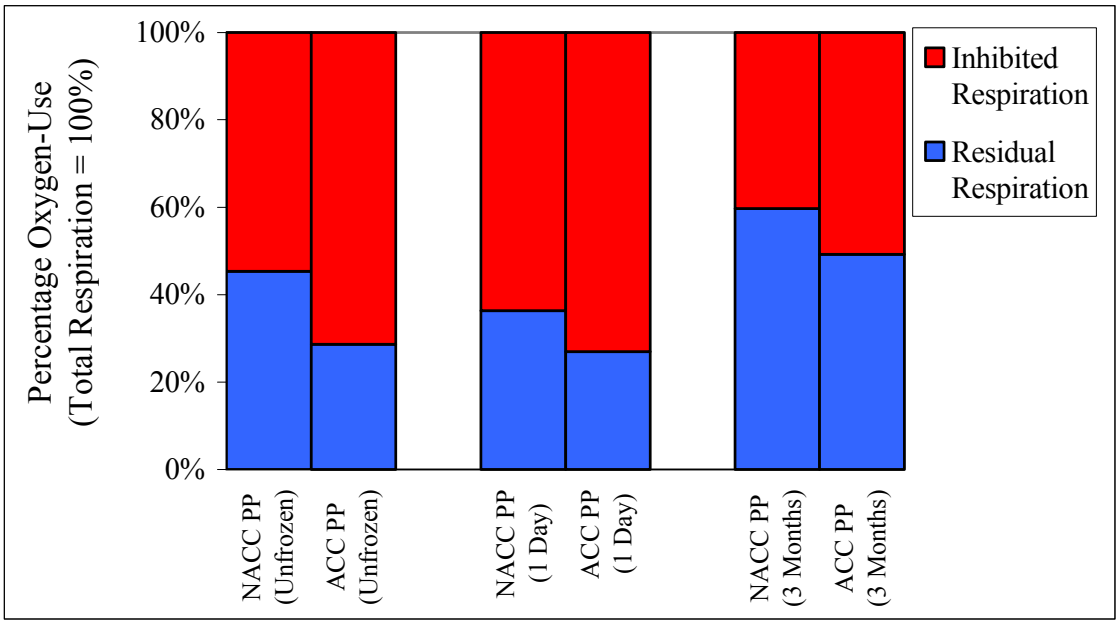



Figure 3.17: Percentage oxygen-use of non-acclimated (NACC) and acclimated (ACC) protoplasts (PP) unfrozen (controls) or frozen for one day or three months in 10% (v/v) glycerol solution and treated with 1 mM KCN and 5 mM SHAM after the completion of a freeze/thaw cycle. Total respiration (= 100%) consisted of inhibited and residual respiration. Inhibited respiration: amount of oxygen-uptake inhibited by protoplast treatment with respiration inhibitors; Residual respiration: amount of oxygen-uptake not inhibited by protoplast treatment with respiration inhibitors.

### *3.5.3 Fungicidal Substance*

The next step was to evaluate the reaction of the biosensor to a substance used in agriculture: Fluazinam, a pyrimidinamine compound. Fluazinam was added to the protoplast suspension (ACC PP frozen in Glyc 10 solution) at seven different concentrations, as indicated in Figure 3.18. Employing fluazinam on its own, reduced the percentage residual respiration only slightly. Increasing the concentration of the substance from 10 nM to 1 mM even lead to a rise of residual respiration. A drop in the percentage oxygen-use was only determined at the very high amount of 10 mM.

To investigate differences in the effects of fluazinam and KCN and/or SHAM on the respiration rate, all three inhibitors were added to the protoplasts, singly or in combination. Adding 5 mM SHAM to the biological units reduced their rate of oxygen-use by 31%. In combination with increasing fluazinam concentrations, the residual respiration rose as well (up to 1 mM fluazinam) (Figure 3.18). No significant differences were detected between the fluazinam only and fluazinam + SHAM. On the other hand, treating the protoplasts with fluazinam and KCN (1 mM), reduced the percentage residual respiration significantly, similar to the effect of fluazinam + SHAM + KCN on the protoplasts. The

addition of fluazinam + KCN to the biological units showed no difference to the KCN-only-control. The two inhibitors revealed a reduced rate of oxygen-uptake with increasing amounts of fluazinam. At low concentrations, the percentage of residual respiration was higher than for the KCN + SHAM-control, whereas no difference were found at high amounts.

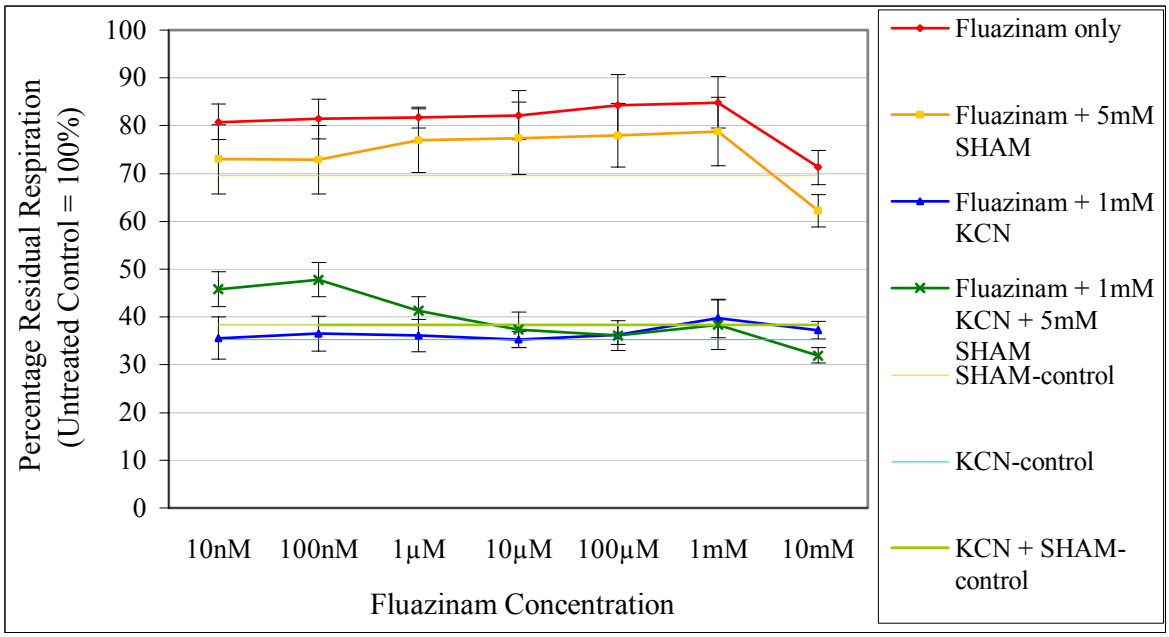



Figure 3.18: Percentage residual respiration of acclimated protoplasts (frozen in 10% (v/v) glycerol solution) after addition of fluazinam at different concentrations with and without 1 mM KCN and/or 5 mM SHAM. The untreated control was set to 100%. The KCN-control (i.e. without fluazinam) had 35.3% oxygen-use (SD: 3.1), the SHAM-control 69.5% (SD: 5.4) and the KCN + SHAM-control 38.3% (SD: 7.5) of the untreated control.

# 4 DISCUSSION

The aim of this study was the development of a procedure to advance the applicability of the protoplasts biosensor under field conditions. Achieving this aim involved three points: An appropriate plant had to be identified from which the mesophyll protoplasts could be acquired. Then a procedure was developed to preserve the activity of the biological units over a longer time period through freezing. This ensures the availability of the biological units at any time. Moreover, a method was elaborated to increase the mechanical stability of the protoplasts through the removal of their vacuoles. Finally, the utilisation and applicability of the frozen protoplasts as the biological units of the biosensor were evaluated. This was accomplished by adding respiration inhibitors and a fungicidal substance used in agriculture to the protoplast suspension.

## 4.1 Validation of the Oxygen-Measurement System

Before employing the oxygen-measurement system its validity had to be determined. It was checked whether different methodological parameters influenced the measurements, possibly falsifying the results. It revealed that the individual electrodes had no significant effects on the measurements; nor did the isolation factors age, length of acclimation, leaf number, and amount of enzyme solution. A time series of measurements over six hours also showed no influence of time on the activity of the protoplasts. In conclusion, the lack of effects of the tested parameters on oxygen-use and -evolution indicated that oxygen-measurements could be used to test for influences of other factors on the protoplasts under defined conditions.

Post-isolation parameters, such as photosynthesis, respiration or other physiological activities, provide reliable information on the degree of cellular integrity and the functional status of protoplasts (Bornman and Bornman, 1985; Hampp *et al.*, 1986). These characteristics are being utilised in the biosensor to determine the presence of toxic substances. Furthermore, the oxygen-measurements were employed in this study as a quality criterion to test the biosensor properties of the protoplast during the three processes outlined above.

## 4.2 Optimisation of Protoplast Isolation

The first step in obtaining an adequate biological component for the biosensor, was to choose a plant for protoplast isolation and optimise the procedure. Protoplasts were derived from *Vicia faba* and *Helianthus annuus*. Previous investigations on the protoplast biosensor were carried out with *V. faba* mesophyll protoplasts (Lindner *et al.*, 1992; Overmeyer *et al.*, 1994a, 1994b) (Chapter 1.4). This presents a basis for further research to build upon. *H. annuus* has been employed in numerous other investigations, including in this Institute (Voeste, 1991; Hutter, 1992; Henn, 1997; Binsfeld, 1999). Therefore, much experience was available on protoplast isolation, as well as on plant growth, even under sterile conditions, processes which were essential for this study. The two plants were compared with regard to the protoplast yields and amount of chlorophyll per g fresh weight leaf material, as well as the biosensor properties of the protoplasts.

With regard to the utilisation of the protoplasts as biological units of the biosensor, the isolation of protoplasts had to be optimised to enable carrying out the isolation and oxygen-measurements on the same day. This was of importance, as storing protoplasts for more than 12 h have led to the regeneration of a cell wall (Upadhya, 1975; Gallbraith, 1981) and a marked decreased in viability. Schnabl and Zimmermann (1989) reported of an increase in ethane evolution, indicating cell disorder and loss of compartmentalisation within 48 h. Protoplasts showed degradation of chlorophyll a and b pigments and changes of key enzymes, such as neutral protease and ribulose biphosphate carboxylase, were elucidated. Thus, it was important to keep the time interval between protoplast isolation and oxygen-measurements low.

### *4.2.1 Isolation of Vicia faba Mesophyll Protoplasts*

To carry out the isolation and oxygen-measurements on the same day, thus preserving the biosensor properties of the protoplasts and avoiding the regeneration of a cell wall, the leaves were incubated in enzyme solution over night. This meant having to change the standard protoplast isolation procedure (Lindner *et al.*, 1992), which included a 4 h incubation of the leaves in enzyme solution to a procedure with 16 h. To avoid bacterial contamination and associated damage of the protoplasts during the new, 16 h incubation at 25°C, the antibiotics kanamycin and cefotaxime were added to the digestion medium. Although a toxicity has been reported for the two antibiotics on wheat cells (Simmonds and Grainger, 1993) and zygotic embryos of white spruce (Tsang *et al.*, 1989), no differ-

ential effect were determined on the number of *Vicia faba* mesophyll protoplasts, when employing the two concentrations of 10 µg $mL^{-1}$ and 50 µg $mL^{-1}$). To nevertheless reduce possible adverse effects of the antibiotics on the protoplasts, the lower concentrations were used during further isolations.

The two incubation times (4 h and 16 h) were compared with regard to protoplast yield, amount of chlorophyll per g fresh weight leaf material, oxygen-use and -evolution (Chapter 3.2.1). Of these parameters only the latter was significantly different for the two procedures, being higher for the 4 h than the 16 h incubation. This may have been the result of harm caused to protoplasts during the isolation procedure e.g. by sanding off the epidermis of the upper leaf surface. Such mechanical stress can damage cells, causing the release of hydrolytic enzymes and phenolic compounds such as tannins, alkaloids and terpenes into the enzyme solution. These in turn may damage organelle membranes: phenolic compounds interact strongly with membrane proteins and lipolytic acylhydrolases with membrane phospholipids (Neuburger, 1985). This may have been the reason for the higher oxygen-evolution of protoplasts isolated using the 4 h incubation of leaf material in the enzyme solution compared to the 16 h incubation.

The release of toxic factors through cell wall removal (Hahne and Lörz, 1988) can lead to an alteration in oxygen balance, resulting in an over-production of reactive oxygen species (ROS), namely superoxide, hydrogen peroxide, and hydroxyl radicals (de Marco and Roubelakis-Angelakis, 1996). Wang and Jia (1994) measured an increase in superoxide dismutase and catalase activities in response to enzyme treatment of wheat mesophyll protoplasts. Biedinger *et al.* (1991) showed that an addition of the radical scavenger vitamin E (tocopherol) protected *V. faba* protoplasts from radical damage during electromanipulation, therefore enhancing their potential viability. In view of possible ROS damage to protoplasts during isolation, tocopherol was added to the enzyme solution, after clean-up or before evacuolation of the protoplasts. However, no increased rates of oxygen-use or -evolution were determined. It seems, therefore, that the concentration of ascorbic acid employed in the two procedures was sufficient to scavenge the released free radicals.

Despite the somewhat lower oxygen-evolution, the protoplasts of the new isolation method could be used as biological units for the biosensor. The great advantage was the possibility of employing the protoplasts for the oxygen-measurements shortly after they were isolated. Thus, a loss of biosensor properties and the regeneration of the cell wall were prevented from occurring during an over-night incubation.

### *4.2.2 Isolation of Helianthus annuus Mesophyll Protoplasts*

Similar to *V. faba*, the isolation procedure for *H. annuus* protoplasts was set out to include an over-night digestion, for the reasons stated above. Two procedures for *H. annuus* protoplasts isolation were compared, one employing sterile plants and the other non-sterile ones. The latter avoided the use of antibiotics by incubating the leaves for 16 h at 4°C. Work on *H. annuus* regeneration in another research group of this Institute showed that the use of antibiotics during the isolation of protoplasts reduced their vitality (unpublished data).

Comparing the two procedures revealed differences in protoplast yield, amount of chlorophyll per g leaf material, oxygen-use and -evolution (Table 3.4). All were significantly higher for the non-sterile protoplasts than the sterile ones. In contrast, the amounts of chlorophyll within the sterile and non-sterile protoplasts were not significantly different. Therefore, although the two plant systems showed no variations in the amount of chlorophyll within the protoplasts, the oxygen-evolution was about 8.9 times less for the sterile than the non-sterile ones. Consequently, the chlorophyll in the sterile protoplasts must have been less active than in the non-sterile ones.

The differences observed between sterile and non-sterile plants may have been the result of ethylene production in the closed culture vessels. The inhibition of gaseous exchange within such containers may have led to the accumulation of ethylene, resulting in abnormal phenomena (Sarkar, 1999), including irregularly shaped shoots, curling leaves (Righetti, 1996), and leaf senescence (Podwyszynska and Goszczynska, 1998). Moreover, a low viability of protoplasts has been correlated with high ethylene production (Rethmeier *et al.*, 1991). Ethylene treated leaves have been shown to have a decreased electron transport capacity in the photosynthetic membranes (Wullschleger *et al.*, 1992). The rate of net photosynthesis improves when growing plants under conditions of through-flow ventilation than in sealed culture vessels, the phenomenon being connected to the presence of ethylene (Zobayed *et al.*, 1999). Thus, the accumulation of ethylene was probably responsible for the lower yield and viability of the sterile protoplasts.

The non-sterile *H. annuus* protoplasts were used in further studies for the following reasons: First of all, the non-sterile plants gave a greater yield than the sterile ones. High numbers of protoplasts were important for the development of a procedure to protect the activity of the biological unit of the biosensor over longer time periods. Moreover, the protoplasts of the non-sterile plants had significantly higher rates of oxygen-use and

-evolution than of the sterile plants, indicating a higher viability. Thus, the non-sterile *H. annuus* plants were more adequate to obtain protoplasts than the sterile plants.

### *4.2.3 Comparison between Vicia faba and Helianthus annuus*

The aim of comparing *V. faba* and *H. annuus* was to determine which plant was more appropriate to provide mesophyll protoplasts. A high protoplast yield and activity was deemed essential for the biological unit for the biosensor. The protoplast yield of the non-sterile *H. annuus* was almost five times higher than for *V. faba*. The considerably higher numbers of protoplasts obtained from *H. annuus* were fundamental to develop a freezing procedure in order to preserve the activity of biological units. Moreover, the *H. annuus* protoplasts had the same rate of oxygen-uptake and a higher rate of oxygen-evolution than the *V. faba* protoplasts. Nevertheless, protoplasts of both plants were used during evacuolation to determine whether one yielded higher numbers and vitality of the recovered mini-protoplasts than the other.

## 4.3 Evacuolation

An increased mechanical stability and a high sensitivity to toxic substances, reducing the detection limit, are desirable characteristics for the biological units of the biosensor, which aims at detecting contaminants. Evacuolation has been reported to increase the mechanical stability of protoplasts (Burgess and Lawrence, 1985). In accordance, the removal of the vacuole would reduce possible damage to the biological unit of the biosensor by pipetting and stirring. Moreover, evacuolated protoplasts were described as being more sensitive to mutagens and toxic substances than normal protoplasts. Since the vacuole is a storage site for toxic materials, its removal means that these substances remain in the cytoplasm, where they can exert their toxic effects (Griesbach and Lawson, 1985). Thus, to take advantage of these traits *V. faba* and *H. annuus* protoplasts were evacuolated and the results compared.

The evacuolation procedure by Griesbach and Sink (1983) had to be adapted to the use of a fixed angle instead of a swing-out rotor, while still attaining the required separation of bands containing the desired mini-protoplasts (MPP) and the undesired cell debris. The final procedure for the evacuolation of *V. faba* protoplasts used a 1:1 dilution of percoll buffer with 0.6 M mannitol solution (Chapter 2.3.1) (Figure 3.6), achieving a recovery of

65.1% MPP, an oxygen-use of 74.8% and an oxygen-evolution of 69.2% of the employed protoplasts. In contrast, no band separation was obtained when employing this method for the evacuolation of *H. annuus* protoplasts. A new procedure, consequently, had to be developed to remove the vacuoles from *H. annuus* protoplasts. Different gradients and dilutions of percoll buffer were tested (Chapter 2.3.2). The implementation of a pre-centrifugation step at 4°C immediately before evacuolation lead to a successful separation of MPP and debris. The method resulted in a MPP recovery of 63.0%, 66.1% oxygen-use and 72.0% oxygen-evolution compared to the normal protoplasts. For both methods it was determined that protoplast loading (i.e. number of PP $mL^{-1}$ and number of mL $tube^{-1}$) did not affect MPP yield or vitality significantly. The evacuolation of *V. faba* and *H. annuus* protoplasts did also not give significantly different results between the two plants, when considering the percentage recovery, oxygen-use and -evolution.

The results obtained in this study are comparable to those stated in the literature: Griesbach and Sink (1983) and Griesbach and Lawson (1985) achieved 100% evacuolation of petunia protoplasts, while Frohnmeyer *et al.* (1994) reported of a yield of 40-50% of parsley protoplasts. Moreover, Griesbach and Sink (1983) found a MPP viability of 40% compared to 60% for normal protoplasts. Hörtensteiner *et al.* (1992) reported of an oxygen-evolution of 60% for tobacco MPP and 20% for barley MPP, compared to normal protoplasts' photosynthesis. The percentage recovery and viability of MPP yielded by the optimised procedures employed in this study, were therefore similar to, sometimes even higher than, the results reported in the literature.

The elaborated evacuolation methods for *V. faba* and *H. annuus* protoplasts developed in this study, consequently, gave excellent results when compared to the data reported in the literature. Having been developed at the laboratory scale, relatively low numbers of protoplasts could be evacuolated when considering large-scale production. Practical considerations led to the conclusion that developing a new procedure to scale-up the production of MPP was beyond the scope of this project. As higher numbers of biological units were needed for the freezing investigations, non-evacuolated, normal protoplasts were employed to acquire a method with which the units' activity could be preserved over longer time periods. Transferring the obtained results of freezing and respiration inhibitor investigations to MPP and increasing the production in scale can set the basis for a future project. First investigations demonstrated the feasibility of such a transfer of results (Chapter 4.5.2).

## 4.4 Protection of Protoplast Activity

The next step in obtaining adequate biological units for the biosensor was to develop a technique for storage of the protoplasts to protect their activity and suspend the regenerative development of the cell wall, making them available whenever needed. Preserving the biological units through freezing allowed their use without the need of isolation immediately before oxygen-measurements. As indicated in the literature review (Chapter 1.5) freezing, however, presents its difficulties due to various forms of injury, which can occur during a freeze/thaw cycle. It is thus of paramount importance to protoplast survival to protect them from freezing damage. Two possibilities exist to achieve protection: cold-acclimation of the plants and addition of cryoprotecting substances to the freezing media.

### *4.4.1 Cold-Acclimation*

Cold-acclimation has been shown to induce metabolic changes in plants, leading to a greater tolerance to freezing conditions (Steponkus *et al.*, 1983; Meryman and Williams, 1985; Wanner and Junttila, 1999). Intracellular solutes with known cryoprotective activity (e.g. sugars, proline, soluble proteins, organic acids) have been found to accumulate in the cytosol of acclimated cells (Grout, 1995; Ouellet *et al.*, 2001). Changes in membrane composition have also been revealed, with lipids becoming more unsaturated with acclimation, leading to the retention of membrane fluidity at lower temperatures (McKersie, 1996a; Taiz and Zeiger, 1998). Since cold-acclimation increases the resistance of the plasma membrane to mechanical stresses, a greater stability of the protoplasts may be achieved (Steponkus, 1984). Consequently, acclimated protoplasts can survive freezing to lower temperatures than non-acclimated protoplasts (Dowgert and Steponkus, 1983; Steponkus *et al.*, 1983).

#### *4.4.1.1 Cold-Acclimation of Helianthus annuus*

In this study, *H. annuus* plants were acclimated to cold conditions (Chapter 2.1.1) for three weeks. This resulted in phenotypical changes of the leaves, which were more yellow (Figure 3.9) than when left to grow at warmer temperatures (20°C). In accordance, the amount of chlorophyll per g. f. wt. leaf material was lower in the ACC than the NACC plants, the ACC PP being less densely packed with chloroplasts than the NACC ones (Figure 3.10). Klimov *et al.* (1990) found that ACC plants contained reduced granal

thylakoids than control plants. Thylakoids have even been reported to swell and distort after acclimation to low temperature (Kratsch and Wise, 2000).

The yield of mesophyll protoplast isolation was also higher for NACC than ACC plants. Bartolo *et al.* (1987) reported of an increased resistance of cold-acclimated cells to hydrolytic enzymes, manifested in reduced cell wall digestibility. Acclimation apparently induces changes, which alter the cell wall, leading to the reduction of protoplast release during isolation. Biochemical changes which may account for this property include phenolic crosslinking between polymers (Bartolo *et al.*, 1987) and wall depositions of lipid and extensin (Weiser *et al.*, 1990). A reduction in cell wall digestibility after cold-acclimation may explain the lower protoplast yield of ACC leaves.

Generally, a lower yield and amount of chlorophyll after acclimation are not beneficial to the biological unit of the biosensor. Yet, other characteristics can be more important than a high yield. As it was the aim of this study to preserve the activity of the biological units, an increased tolerance to cold conditions is of importance. It was of interest to investigate the effects of cold-acclimation on evacuolation and freezing of protoplasts.

#### *4.4.1.2 Cold-Acclimation and Evacuolation*

Combining the two procedures (acclimation and evacuolation) may allow the combination of the desired effects of both processes within the biological unit, i.e. an increased tolerance to freezing conditions due to the cold-acclimation and a higher mechanical stability resulting from evacuolation. A comparison of the rates of oxygen-use and -evolution between NACC and ACC MPP and their respective protoplasts was carried out (Chapter 3.4.1.1). The rate of oxygen-evolution showed a clear difference between the NACC and ACC MPP. The NACC MPP had significantly lower rates than their respective protoplasts. This was not the case for the ACC MPP: the rate of oxygen-evolution was the same for normal protoplasts and those which had their vacuoles removed. The rate of oxygen-use, on the other hand, was lower after evacuolation, regardless of whether the protoplasts were acclimated or not. The ACC MPP showed even less oxygen-consumption than the NACC MPP, standing in clear contrast to the results obtained when measuring oxygen-evolution. Thus, differences were evident for the rates of oxygen-use and -evolution measured between NACC and ACC MPP.

A direct comparison of these results with those reported in the literature was not possible, since no studies could be found, which investigated the effects of acclimating plants to

cold conditions on the evacuolation of their protoplasts. Thus, the findings of investigations on the separate procedures had to be employed. Hörtensteiner *et al.* (1992) reported of a 40% reduction of photosynthesis for tobacco protoplasts and an 80% reduction for barley protoplasts through evacuolation. Since these protoplasts were isolated from non-acclimated mesophyll tissues, the results correspond well with those obtained in this study. In contrast, the oxygen-evolution of protoplasts from acclimated leaves were not reduced when the vacuoles were removed. This indicated that changes must have occurred during cold-acclimation, which made the chloroplasts more tolerant of the evacuolation procedure compared to NACC PP. Numerous studies have presented the adaptation of the photosynthetic apparatus to low temperatures (Nyuppieva *et al.*, 1984; Klimov *et al.*, 1990; Artus *et al.*, 1996; Strand *et al.*, 1999). Uemura and Steponkus (1997), for example, showed that acclimating winter rye to cold conditions for four weeks resulted in a significant alteration of the inner and outer chlorophyll membranes. Such changes are likely to be responsible for an increased tolerance of chloroplasts to cold conditions and may have protected them during the evacuolation procedure.

In contrast, the rate of oxygen-use was lower after the removal of the vacuoles, the ACC MPPs' rate being even more reduced than the NACC MPPs'. Frohnmeyer *et al.* (1994) reported of a decrease in the number of mitochondria of 28% in evacuolated parsley protoplasts, as compared to the normal ones. Such a reduction in mitochondria would inevitably have reduced the rate of oxygen-use. Mitochondria have been reported to grow in size during cold-acclimation, to ensure the maintenance of energy supply at low temperatures (Kislyuk *et al.*, 1995). This may have increased their loss in ACC protoplasts during evacuolation, compared to NACC ones, which contained smaller mitochondria.

In conclusion, the acclimation of *H. annuus* plants to cold conditions did influence the evacuolation of protoplasts. The results indicate that the acclimation procedure may have altered the chloroplasts, making them more tolerant against mechanical stress during the evacuolation procedure. Thus, no loss in chlorophyll activity was measured, presenting a clear advantage over the NACC MPP. The rate of oxygen-use, on the other hand, was reduced when combining the two procedures. This could have been brought about by an increased loss of mitochondria during the evacuolation procedure, instead of damage to the MPP. Consequently, the lower rate of oxygen-use did not indicate injury to the mitochondria, an important finding for maintaining the biosensor properties.

This study could only scrape the surface of the complex changes which occur during cold-acclimation. Thus, further studies are needed to elucidate the effects of cold-acclimation

on the various cell components and how these changes affect evacuolation. Due to the relatively low number of protoplasts which could be evacuolated, protoplasts with vacuoles had to be used for further investigations. The obtained results can, however, set the basis for a future study to transfer these results to MPP, in order to increase the mechanical stability of the biological unit of the biosensor, as well as their sensitivity to toxic substances.

#### *4.4.1.3 Cold-Acclimation and Freezing*

As described above, acclimating plants to cold condition has been reported to increase their tolerance against freezing injury (Steponkus *et al.*, 1983; Steponkus, 1984; Meryman and Williams, 1985; Wanner and Junttila, 1999). The question to be investigated was, whether cold-acclimation affected the activity of the potential biological units of the biosensor before and after freezing at -20°C.

The results presented clear evidence that cold-acclimation affected oxygen-evolution (Figure 3.11). Only the ACC PP still showed photosynthesis after 45 minutes of freezing. The rate of oxygen-evolution depended strongly on the media in which the protoplasts were frozen. The results will be discussed further in chapter 4.4.2.1.

In comparison to oxygen-evolution, oxygen-use was still measurable when freezing over much longer time periods (Chapter 3.4.2). Saradadevi and Raghavendra (1994) reported that the photosynthetic apparatus is damaged more easily than respiration. Maintaining respiration activity after freezing was a great success for the applicability of the biosensor, as it allowed the storage of the biological units over longer time periods, ensuring their availability whenever needed.

After revealing that respiration remained active after the completion of a freeze/thaw cycle, appropriate biological units had to be identified, i.e. units which indicated the least injury. Comparing the rate of oxygen-use of unfrozen NACC and ACC protoplasts revealed that they did not differ significantly from each other (Table 3.6). In contrast, the NACC units showed significantly higher rates of oxygen-use after freezing than ACC ones, regardless of the media in which they were frozen (Figure 3.12). A higher uptake of oxygen may be indicative of stress and damage to the biological units.

The elevated oxygen-use of NACC, as compared to ACC PP, may have been the result of an oxidative burst. Plant cells have been shown to initiate oxidant production upon stress

(Prasad *et al.*, 1994). Such stress may be mechanical, such as stirring (Legendre *et al.*, 1993), or may be caused by distortions of the plasma membrane during freeze/thaw injury or accompanying water stress (Yahraus *et al.*, 1995). During chilling, for example, critical changes can occur in membrane fluidity. Any phase changes in the inner mitochondrial membrane have drastic effects on the orientation of the electron transport chain components, resulting in an increase in ROS formation (Creencia and Bramlage, 1971). ROS can accumulate to damaging concentrations, causing oxidative damage to the inner mitochondrial membrane (Kowaltowski *et al.*, 1998; Kowaltowski, 2000). This has been reported especially in NACC cells, because of low levels of alternative oxidase and antioxidant enzymes (Prasad *et al.*, 1994). Acclimation of plants leads to an increase in the activity of the alternative oxidase (Wagner and Krab, 1995; Popov *et al.*, 1997), which avoids ROS formation. Moreover, the amount of antioxidants (Leipner *et al.*, 2000; Munné-Bosch and Alegre, 2000) and antioxidant enzymes, such as catalase 3 and peroxidase, is increased during acclimation. These reduce the numbers of ROS, which have nevertheless been formed (Prasad *et al.*, 1994; Zeng *et al*, 1994; Badiani *et al*, 1997; Prasad, 1997). Preventing the accumulation of ROS protects acclimated plants from oxidation of proteins and lipids during chilling stress and recovery (Prasad, 1996).

Cold-acclimation has also been shown to change the behaviour of the plasma membrane (Steponkus 1984, 1985) through alterations in membrane composition (Dowgert and Steponkus, 1984; Yang *et al*, 1986; Loubaresse and Dereuddre, 1990). These include an incorporation of a higher proportion of unsaturated fatty acids, leading to the maintenance of membrane fluidity at lower temperatures (Chen *et al.*, 1994; Taiz and Zeiger, 1998). As a result, fewer ROS are generated and less damage occurs (Queiroz *et al.*, 1998) in ACC cells, as compared to NACC ones.

Therefore, the NACC protoplasts' higher rate of oxygen-use, compared to those of the ACC ones, was most likely due to increased ROS formation and indicated damage. This finding was supported by the results obtained when freezing the protoplasts for three months. After this period the ACC units showed a much lower loss of activity than the NACC ones. The remaining percentage oxygen-use of the three month old biological units was 61% for the NACC and 70% for the ACC ones, compared to their respective unfrozen controls (Figure 3.13). This clearly strengthened the inference that the acclimation procedure led to a higher protection of the biological units' activity.

In conclusion, the higher rate of oxygen-uptake of NACC units after freezing indicates that they incurred more damage than the ACC ones, which were protected against

freeze/thaw injury by alterations induced during cold-acclimation. This finding is very important to the biosensor development. Cold-acclimation clearly protects the biological units from damage, preventing loss of activity. The use of freezing to store them for longer time periods is thus made possible, making them available whenever needed.

### *4.4.2 Cryoprotectants*

The second method to protect the biological units from freezing injury was the addition of cryoprotectants to the freezing media. The choice of which substance(s) should be used was difficult due to the vast number of publications around the topic of cryoprotection. Valuable lessons on cryoprotectants have been learned from studying organisms which acquired tolerance to cell dehydration from freezing, drying, or the exposure to hyperosmotic environments (Meryman and Williams, 1985). Consequently, numerous compounds have been found to exert cryoprotection, including sugars (Santarius, 1973; Lineberger, 1980; Strauss *et al.*, 1986), proline (Steponkus, 1984; Rudolph *et al.*, 1986; Anchordoguy *et al.*, 1987), glycine betaine (Coughlan and Heber, 1982; Holmström *et al.*, 2000), hydroxyethyl starch (Farrant *et al.*, 1977; Crowe *et al.*, 1997), glycerol (Rowe, 1966; Mazur, 1970), and antifreeze proteins (McKersie, 1996a; Hiilovaara *et al.*, 1999; Yu and Griffith, 1999); their chemical diversity being "bewildering" (Finkle *et al.*, 1985). Most investigations have been restricted to a few of these cryoprotectants (Carpenter and Crowe, 1988). Differences in protection have been reported for the given compounds used with varying cell types and under dissimilar cooling rates (Doebbler, 1966), increasing the difficulties in comparing the effects of these substances.

To protect the protoplasts from freezing injury, the viscous protectant glycerol and the non-reducing dissaccharide trehalose were employed. Trehalose prevents dehydration-induced membrane fusion (Steponkus, 1984) by acting as replacement for water and maintain the hydrophobic-hydrophilic orientation of the phospholipids. Water loss or low temperature may alter the organisation of membrane lipids. The formation of a gel or hexagonal$_{II}$ phase leads to changes in the association between proteins and lipids, permeability, and solute transport across the membrane. Preventing such phase transitions is important as otherwise discontinuities are caused in the membrane, resulting in the leakage of cytoplasmic solutes and the disruption of membrane enzyme complexes (McKersie, 1996b). Furthermore, Anchordoguy *et al.* (1987) reported that trehalose was markedly better than sucrose at preserving membranes and liposomes. Therefore, treha-

lose was one of the compounds chosen to protect the protoplasts during the freeze/thaw cycle.

The other cryoprotectant was glycerol, one of the most widely used protecting agents. Finkle *et al.* (1985) classed glycerol in the group of compounds with the highest protection action against freezing injury. Glycerol reduces the rate of water diffusion out of the cell as the solution becomes more concentrated with decreasing temperature (Meryman and Williams, 1985). The electrolyte concentration in the residual unfrozen solution in and around the cell is consequently reduced (Mazur, 1970). Compared to dimethylsulphoxide (DMSO), a commonly used cryoprotectant, glycerol has the advantage of being non-toxic at elevated concentrations (Meryman and Williams, 1985; Fahy, 1986). These characteristics led to the choice of glycerol as the other cryoprotectant used in this study.

#### *4.4.2.1 Freezing Helianthus annuus Mesophyll Protoplasts*

Four freezing media were employed containing 0.4 M trehalose (Treh 4), 0.6 M trehalose (Treh 6), 5% (v/v) glycerol (Glyc 5), or 10% (v/v) glycerol (Glyc 10) (Chapter 2.6). These media greatly influenced the rates of oxygen-evolution after completion of a freeze/thaw cycle (Figure 3.12). After 30 minutes freezing, the protoplasts in the glycerol media still produced oxygen. While the ACC PP also showed photosynthetic activity in the trehalose solutions, none could be measured for the NACC ones. Of all combinations, only the ACC PP in Glyc 10 still photosynthesised after freezing over a period of 45 minutes. This demonstrates that both processes, cryoprotection and acclimation, affected photosynthesis.

The drop in temperature with time was probably responsible for the reduction or even loss of photosynthetic activity. Steponkus *et al.* (1998) found that below -4°C injury of protoplasts is manifested as a loss of osmotic responsiveness, resulting from freeze-induced lamellar-to-hexagonal II ($H_{II}$) phase transition (Chapter 1.5.1). This is an interbilayer event, observed most often in regions where the plasma membrane comes into close contact with the chloroplast envelope as a result of freeze-induced dehydration. Bilayers are reformed upon rehydration upon thawing. Yet, integral proteins can be lost from the membrane or reassociate with the bilayer in a way that is inconsistent with normal function (Crowe and Crowe, 1982). Thus, the plasma membrane is likely to become meddled with the endomembranes, especially the chloroplast envelope (Steponkus *et al.*, 1998).

This was probably the reason why a loss of photosynthetic activity was observed with increasing time.

Freeze-induced formation of the $H_{II}$ phase can be deferred to lower temperatures. Processes responsible for this phenomenon include the alterations of membrane lipid composition and the accumulation of sugars during acclimation (Steponkus *et al.*, 1998). In accordance, several research groups have reported of changes of chloroplasts and adaptations of the photosynthetic apparatus to low temperature (Nyuppieva *et al.*, 1984; Klimov *et al.*, 1990; Artus *et al.*, 1996; Strand *et al.*, 1999) (Chapter 4.4.1). Moreover, the addition of protective compounds have also been reported to inhibit or postpone phase changes (Crowe and Crowe, 1982; Crowe *et al.*, 1983). These phenomena were clearly evident in the results of this study. The combination of acclimation and cryoprotection lead to a higher preservation of photosynthetic activity. The fact that ACC PP still produced oxygen after 45 minutes of freezing in Glyc 10 indicates further, that some optimisation of the freezing regieme will most probably lead to the protection of photosynthesis in addition to respiration. These results, therefore, set a basis for a further study.

Furthermore, the protective action of the four media was tested on the respiration activity of ACC and NACC biological units after completion of a freeze/thaw cycle. No significant differences of the effects of the four media were detected on the rates of oxygen-use for either of the protoplast types. However, a tendency of a higher oxygen-consumption could be seen for NACC PP frozen in the trehalose solutions than for those in the glycerol media. Differences between the media were confirmed when employing inhibitors (Chapter 4.5.2).

## 4.5 Responsiveness of the Biological Units

The responsiveness of the NACC and ACC protoplasts was tested towards substances, which affect respiration to determine the suitability of the frozen protoplasts as biological units of the biosensor in practice. As described in the literature review (Chapter 1.2) the mitochondrial electron transport of plants has two pathways: the normal, cytochrome pathway and the cyanide-resistant, alternative pathway. The two inhibitors KCN and SHAM were used to block electron transfer, the former inhibiting the cytochrome pathway and the latter the alternative pathway. As a result, the electrons could not be passed onto oxygen to form water, consequently reducing the rate of oxygen-consumption. Thus,

the response of protoplasts towards an inhibition of respiration was determined by changes in the rate of oxygen-use.

### *4.5.1 Effects of Respiration Inhibitors on Protoplasts*

Treating the protoplasts with the inhibitors KCN and SHAM, singly or in combination, led to variations in the shapes of the oxygen-measurement curves (Figure 3.14), indicating different effects of the inhibitors on the respiration pathways. As expected, SHAM reduced the rate of oxygen-use of protoplasts, compared to the untreated control. In contrast, KCN led to an initial increase of oxygen-uptake. After about one minute, the inhibition of mitochondrial respiration became apparent, being stronger for KCN than SHAM.

The initial increase in oxygen-consumption within the first minute after adding KCN to the protoplasts can be explained by an accumulation of free electrons within the respiration pathway. Blocking the cytochrome pathway means that electrons can no longer flow through it. Since the alternative pathway does not have the capacity to oxidise all ubiquinol, other electron acceptors have to be used. Instead of being transferred onto the oxidases, the electrons reduce $O_2$ to $O_2^{-\bullet}$ (Popov *et al.*, 1997), generating ROS (Robertson *et al*., 1995) and leading to the increase in oxygen-use. Once the pathway is cleared of electrons, the oxygen-uptake is reduced.

These findings are supported by the effects revealed when employing both inhibitors in combination. Blocking both pathways led to an increase in the rate of oxygen-uptake, which was higher and lasted longer (0-2 min) than when using KCN only. In the presence of the two inhibitors, electrons can no longer be transferred to the cytochrome and alternative oxidases. The resulting accumulation of intermediately reduced ubiquinones leads to an increase in ROS formation, as $O_2$ is reduced to $O_2^{-\bullet}$ (Cadenas *et al.*, 1977) (see Chapter 1.2 and 4.5.2 for more detail of ROS formation). Respiratory inhibitors have previously been shown to increase mitochondrial ROS generation (Purvis *et al.*, 1995; Popov *et al.*, 1997; Braidot *et al.*, 1999). Thus, the addition of KCN ± SHAM to the protoplast suspension initiated an oxidative bust, leading to the characteristic elevated rate of oxygen-consumption within the first one or two minutes after treatment. The rate of oxygen-use was reduced significantly, as the electrons were removed from the respiration pathways.

In contrast to the KCN ± SHAM treatments, no accumulation of electrons occurred when blocking the alternative pathway only, using SHAM. The cytochrome pathway has a higher capacity (i.e. maximum level of activity attainable) than the alternative pathway and can consequently cope with a higher flow of electrons. Thus, an initial increase in oxygen-uptake was not apparent when employing SHAM, as most electrons can flow through the cytochrome pathway.

Looking at the degree of inhibition of respiration brought about by KCN and/or SHAM revealed significant differences (Table 3.7). As expected, SHAM had a lower inhibitory effect on oxygen-consumption than KCN, since the electron flow through the alternative pathway is lower than through the cytochrome one (Ribas-Carbo *et al.*, 1995). Even the addition of both inhibitors to the protoplasts still showed some remaining oxygen-uptake, although both respiration pathways were blocked entirely.

Two possibilities exist, which could explain this so-called 'residual respiration': 1) the respiration was not inhibited 100% or 2) other processes exist, which use $O_2$. The first potential explanation is contradicted by the fact that KCN and SHAM caused different percentages inhibition of oxygen-uptake. For example, the addition of KCN + SHAM to the unfrozen protoplasts led to a residual respiration of 49% for NACC and 29% for ACC protoplasts. Furthermore, a higher percentage residual respiration was revealed after freezing protoplasts for three months (NACC: 60%, ACC: 49%), as compared to one day (NACC: 36%, ACC: 27%) (Figure 3.17). Liang *et al.* (1987) and Sesay *et al.* (1986) also reported of residual respiration rates of 20-30% and 42-44%, respectively, when adding these inhibitors to unfrozen cells. This remaining oxygen-consumption may thus contribute significantly to oxygen-uptake (Ribas-Carbo *et al.*, 1997). Wagner and Krab (1995) also emphasised that a considerable amount of oxygen-consumption was insensitive to respiration inhibitors. The question therefore arises which processes are responsible for such elevated levels of oxygen-uptake, without being blocked by respiration inhibitors.

Non-mitochondrial oxidases have been reported as being responsible for such uninhibited oxygen-uptake (Tukeeva *et al.*, 1994). Liang *et al.* (1987) suggested glycollic acid oxidase within microbodies as the reason for the residual respiration in tobacco callus cultures. Reddy and Srivastava (1998) indicated that oxygenases may be stimulated to accept electrons and reduce oxygen to water. However, residual respiration has also been measured in isolated mitochondria (Ribas-Carbo *et al.*, 1997) and thus other processes responsible for oxygen-uptake must be involved within the mitochondria.

Another possible mechanism for the occurrence of residual respiration is the formation of ROS, such as superoxide, $H_2O_2$, and hydroxyl radicals. Kowaltowski (2000) states that the continuous leak of electrons makes the mitochondria the main generation site for reactive species in most cells. Monoelectronic reduction of oxygen, leading to ROS formation, occurs mainly at the level of complexes I and III of the respiratory chain, most likely through the donation of electrons from intermediately reduced ubiquinones (Cadenas *et al.*, 1977). As indicated in the literature review (Chapter 1.2), ROS generation may be the result of an over-reduction of the mitochondrial respiratory chain components due to the saturation of the cytochrome pathway with electrons (Wagner and Krab, 1995; Vanlerberghe and McIntosh, 1996). This may occur whenever the mitochondrial electron transport is restricted (Maxwell *et al.*, 1999). Not surprisingly, therefore, blocking one or more respiratory pathways using inhibitors may lead to an increase in radical formation (Purvis *et al.*, 1995; Millenaar *et al.*, 1998; Popov and Starkov, 1998). The accumulation of ROS may be exacerbated by SHAM and KCN, as they have been reported to inhibit peroxidases (Maxwell *et al.*, 1999; Papadakis and Roubelakis-Angelakis, 1999), which convert $H_2O_2$ to $H_2O$ and $O_2$ (Heldt, 1986). Thus, a high residual respiration will be indicative of ROS formation, which is not desirable for the biological unit of the biosensor and should consequently be kept low.

Transferring the results obtained using normal PP onto MPP only makes sense, if the evacuolated protoplasts also react towards respiration inhibitors in a similar manner. The percentage inhibition of ACC PP was 71%. Inhibiting respiration of ACC MPP revealed a 73% reduction, being comparable to the result obtained for the normal protoplasts. This indicates that the PP with and without vacuoles responded similarly towards the inhibitors, setting a solid basis for a further study, in which the results obtained using normal protoplasts can be transferred onto MPP.

### *4.5.2 Comparison of the Unfrozen and Frozen Protoplasts' Responses towards Inhibitors*

It was determined whether the potential biological units responded differently towards respiration inhibitors after freezing than before. Comparing NACC and ACC protoplasts showed that the NACC PP (unfrozen and frozen) had a significantly higher rate of oxygen-uptake when employing the inhibitors than the ACC ones (Table3.7; Figure 3.15). This may have been the result of damage to the protoplasts brought about by the oxidative burst, as described in chapter 4.4.1.3.

No significant influence of the media was determined on the rate of oxygen-use without treating the frozen protoplasts with respiration inhibitors (Chapter 4.4.2.1). In contrast, the media in which the protoplasts were frozen had a clear effect on the degree of inhibition of oxygen-uptake (Figure 3.16). The percentage inhibition was lowest for the units frozen in trehalose media. Blocking both respiration pathways entirely only led to a reduction in oxygen-use of around 40%. In contrast, those frozen in glycerol media had the much higher inhibition of respiration of about 70%, the same value as determined for the unfrozen control protoplasts.

These findings were highly important for the biosensor development. First of all, a decrease of respiration of 70% gave a greater range for inhibition than a 40% reduction. This is essential to allow the determination of the degree of inhibition conveyed by an environmental sample. Moreover, the results indicated that the biological units frozen in the glycerol solutions had more similar characteristics to the unfrozen control than those frozen in the trehalose media. Since the control protoplasts did not go through a freeze/thaw cycle, they were exposed to less stress and suffering less damage than the frozen biological units, especially those which showed significant differences to the controls.

The results seem to indicate that the cryoprotective substances had a higher positive effect on the preservation of protoplast activity than the acclimation process. The importance of the acclimating process developed in this study was revealed after freezing the protoplasts for three months (Figure 3.13). After that time period the ACC PP showed less reduction in the rate of oxygen-use than the NACC ones. In addition to the higher preservation of activity of the ACC PP after three months of freezing, they also retained a lower residual respiration compared to the NACC PP (Figure 3.17). This presented clear evidence that a combination of the two developed procedures (cryoprotective medium and cold-acclimation) is of great importance to preserve protoplast activity for longer time periods.

In conclusion, the ACC protoplasts, frozen in Glyc 10 were chosen as the most adequate biological units for the biosensor, since the acclimation of *H. annuus* plants and the use of the Glyc 10 protected the protoplasts highly during the freeze/thaw cycle. This became evident as:

a) ACC protoplasts had a lower residual respiration, indicating less damage than for NACC protoplasts. This was the case for unfrozen protoplasts, as well as those frozen for three months.

b) Protoplasts frozen in Glyc 10 conveyed lower rates of oxygen-uptake and a higher inhibition of respiration. These values were significantly the same as of the unfrozen controls, indicating a non-significant effect of freezing on the activity of the biological units.

Thus, of all combinations (ACC/NACC, freezing media), the ACC protoplasts in 10% (v/v) glycerol solution came closest to the unfrozen control. Moreover, a larger range of possible inhibition was revealed for ACC compared to NACC protoplasts. This greater range means that more differentiation is possible to determine the degree of inhibition induced by substances in environmental samples. ACC protoplasts in Glyc 10 were therefore used to test the effect of the fungicidal compound fluazinam.

### *4.5.3 Fungicidal Substance*

As described above, it has already been shown that the frozen protoplasts do respond towards respiration inhibitors, consequently demonstrating that they are suitable as biological units of the biosensor. Nevertheless, fluazinam was also used to test the applicability of the biosensor. This pyrimidinamine is the active ingredient of the fungicides Shirlan (Zeneca) and Frownicide (Ishihara Sangyo) (Komyoji *et al.*, 1995, Drexler and Stuke, 1999). The fungicidal action of fluazinam is said to influence respiration (Bayer AG, Division of Plant Protection, personal communication). Only one study can be found in the literature, which indicates that fluazinam may possibly have an uncoupling action when using rat liver mitochondria (Guo *et al.*, 1991). Regarding the uncertainty about the mode-of-action of the fungicidal substance, it should be determined whether it could be detected by the biosensor.

Adding different concentrations of fluazinam to the biological units of the biosensor showed only a small reduction in the rate of oxygen-uptake, compared to the untreated control (Figure 3.18). When increasing the amount from 10 nM to 1 mM a rise in oxygen-consumption was determined, as opposed to the expected decrease. Only at the very high concentration of 10 mM was a clear reduction in the rate of oxygen-use apparent. This reaction of the biological units towards fluazinam was surprising, since it was thought to act as an inhibitor of respiration and the inhibitors KCN and SHAM had clearly led to a decline in oxygen-uptake. This indicated that an inhibition of mitochondrial respiration could not be fluazinam's primary mode-of-action.

Uncouplers disconnect ATP synthesis from the electron transport chain by stimulating the permeability of the mitochondrial membrane to protons (Kowaltowski, 2000). Consequently, the transfer of protons across the membrane is no longer dependent on ADP availability and can thus be used continuously to allow a transfer of electrons along the respiration pathways. This stimulatory effect of uncouplers on respiration (Taiz and Zeiger, 1998) could be seen when increasing the levels of fluazinam from 10 nM to 1 mM. These results therefore indicate that fluazinam does indeed have an uncoupling mode-of-action.

The addition of the respiration inhibitors KCN and SHAM further supported these conclusions. Treating the biological units with only KCN and/or SHAM, led to clear reductions in the rate of oxygen-use, as previously described (Chapter 4.5). When SHAM (5 mM) was added to the units in combination with increasing fluazinam concentrations, the oxygen-use was higher compared to the SHAM-only-control. This was also probably brought about by the stimulation of electron transport through the cytochrome pathway in conjunction with an increase in ROS formation. In contrast, such a rise was not detected when employing KCN with increasing amounts of fluazinam. This lack of stimulation of electron transfer was most likely due to an already saturated alternative pathway. These results clearly demonstrate that (at least one of) the mode(s)-of-action was an uncoupling and not an inhibition of respiration.

At the extremely high concentration of 10 mM fluazinam, a decline was found for the oxygen-consumption rate of protoplasts. The reduction was possibly due to a direct effect of the substance on respiration at such a high amount. A similar action has been reported for the pesticide 2,4-dinitrophenol, which has other effects in addition to uncoupling (Caprette, 2000). This decrease in residual respiration at 10 mM fluazinam was also apparent when the inhibitors KCN and SHAM were added to the protoplast suspension.

In conclusion, the protoplast biosensor revealed that fluazinam does not primarily inhibit respiration, but acts as an uncoupler and that the observed inhibition of respiration was a general toxic effect at very high fluazinam concentrations. The protoplast biosensor thus made a valuable contribution to the question of the mode-of-action of fluazinam. The results clearly demonstrated that the protoplast biosensor not only gave evidence of respiration inhibiting substances (KCN and SHAM) but can also indicate the site- and mode-of-action of substances.

# 5 SUMMARY

The aim of this study was to develop a method to advance the applicability of the biosensor under field conditions. In accordance, first an appropriate donor plant had to be identified to provide the protoplasts. The sunflower (*Helianthus annuus*) was most adequate, as it reached higher yields of vital mesophyll protoplasts than the broad bean (*Vicia faba*).

The biggest problem for the application of the biosensor in the field was the loss of protoplast viability within a relatively short time span leading to the loss of the biosensor properties. A method was thus developed, which prolonged the time over which the protoplasts could be used as biological units of the biosensor. It was shown that the properties of the protoplasts were maintained after the development of appropriate freezing conditions showing a clearly measurable respiration, which could be blocked by respiration inhibitors.

That the frozen protoplasts were suitable for the use as biological units of the biosensor was the result of having developed special protocols, to reduce the damage of the units during the completion of a freeze/thaw cycle. Acclimating sunflower plants to cold conditions led to an increased preservation of the biosensor properties of the biological units after freezing. Another important step in optimising the freezing process was the development of the freezing medium. Frozen, as well as unfrozen protoplasts involved a certain amount of oxygen-use, which was not the result of cytochrome or alternative pathway respiration. This residual respiration was probably due to the generation of reactive oxygen species, which can damage the biological units and thus needs to be kept to a minimum. The use of glycerol in the freezing medium revealed a significant reduction of residual respiration compared to the trehalose solution, the levels being the same as for the unfrozen protoplasts. The developed protocols for *H. annuus* plant growth and protoplast freezing led to a clearly measurable respiration after freezing them in 10% (v/v) glycerol solution for three months. Consequently, these protoplasts were adequate to be used as biological units of the biosensor.

It is expected that the freezing protocol can be optimised further by using evacuolated protoplasts (MPP), which have a higher mechanical stability than normal protoplasts. First experiments on the removal of the vacuoles yielded extremely promising results on the recovery and vitality of the MPP. Yet, the technical equipment of the laboratory was not

set out to evacuolate large numbers of protoplasts, which were needed to test the freezing protocol. The results obtained by using protoplasts can, however, be transferred to MPP, as first investigations on the inhibition of respiration demonstrated.

Using the respiration inhibitors KCN and SHAM clearly demonstrated the suitability of the frozen protoplasts as biological units of the biosensor in practice. A further substance, which is being utilised in agriculture was nevertheless employed. Fluazinam is a compound with fungicidal properties, acting upon respiration. It was revealed that unlike KCN or SHAM, fluazinam did not inhibit respiration but acted as an uncoupler. Thus, in addition to giving evidence of respiration inhibiting substances, the protoplast biosensor can also indicate the site- and mode-of-action of substances.

The results obtained in this study contribute substantially to stabilising the biosensor properties of the protoplasts as the biological units of the biosensor over longer time periods. The biological units are, consequently, made available for use at any time. This presents a clear progress towards employing the protoplast biosensor routinely in the field to detect contaminants, including respiratory inhibitors.

# ZUSAMMENFASSUNG

Ziel der Arbeit war es, die Verwendbarkeit der Protoplasten als biologische Einheit des Protoplastenbiosensors im Hinblick auf eine Feldtauglichkeit des Biosensors hin zu verbessern. Hierzu wurde im ersten Schritt eine geeignete Protoplastenspenderpflanze ausgewählt. Als am besten geeignet hat sich hierbei die Sonnenblume (*Helianthus annuus*) herausgestellt, da diese im Vergleich zur Ackerbohne (*Vicia faba*) eine wesentlich größere Ausbeute an vitalen Mesophyll-Protoplasten ergab. Im nächsten Schritt wurde dann eine Methode entwickelt, die eine verlängerte Verwendbarkeit der Protoplasten als biologische Einheit ermöglicht, da die geringe Lebensdauer und damit der Verlust der sensorischen Eigenschaften der Protoplasten bisher das größte Hindernis für den praktischen Einsatz des Protoplastenbiosensors darstellt. Es konnte gezeigt werden, dass bei geeigneter Wahl der Bedingungen über ein Einfrieren der Protoplasten deren biosensorische Eigenschaften soweit erhalten bleiben, dass sie nach dem Auftauen als biologische Einheit verwendet werden können. So zeigten die Protoplasten nach dem Auftauen eine deutlich messbare Atmung, die sich über die Zugabe von Atmungsinhibitoren hemmen ließ.

Zur Optimierung des Einfrierprozesses wurde ein spezielles Anzuchtprotokoll für die Sonnenblumen entwickelt, da gezeigt werden konnte, dass in tiefgefrorenen Protoplasten aus kälteakklimatisierten Sonnenblumen die biosensorischen Eigenschaften wesentlich besser konserviert werden, als in nicht kälteakklimatisierten. Ein weiterer wesentlicher Schritt zur Optimierung des Einfrierprozesses bestand in der Entwicklung eines Einfriermediums. Durch die Verwendung von Glycerin im Medium konnte gegenüber der Trehalose-Variante eine drastische Senkung der Restatmung erreicht werden, die dann ca. die Größenordnung der nicht eingefrorenen Protoplasten hatte. Sowohl die nicht eingefrorenen Protoplasten, als auch die eingefrorenen Protoplasten, wiesen einen nicht auf die cytochromale bzw. alternative Atmungskette zurückzuführenden Sauerstoffverbrauch auf. Diese Restatmung ist wahrscheinlich auf die Bildung reaktiver Sauerstoffspezies zurückzuführen, die die biosensorischen Eigenschaften des Protoplasten schädigen und daher niedrig gehalten werden müssen. Die in Glycerinmedium 10 eingefrorenen Protoplasten akklimatisierter Sonnenblumen wiesen auch nach dreimonatiger Lagerung noch eine deutlich messbare mitochondriale Atmung auf und konnten als biologische Einheit des Protoplastenbiosensors eingesetzt werden.

Es ist zu erwarten, dass sich der Einfrierprozess durch die Verwendung evakuolisierter Protoplasten, die eine deutlich höhere mechanische Stabilität besitzen, weiter optimieren lässt. Erste Experimente zur Evakuolisierung von Sonnenblumenprotoplasten ergaben, bezogen auf die Ausbeute evakuolisierter Protoplasten (MPPs), deren Vitalität und Hemmbarkeit der Atmung, sehr vielversprechende Ergebnisse. Die technische Ausrüstung des Labors war jedoch nicht darauf ausgelegt, größere Mengen an MPPs zu isolieren, die benötigt worden wären, um das für Protoplasten entwickelte Gefrierprotokoll auch an MPPs zu testen. Die an Protoplasten gewonnen Erkenntnisse lassen sich jedoch auf MPPs übertragen.

Schon durch den Einsatz der Atmungsinhibitoren KCN und SHAM konnte die Praxistauglichkeit der eingefrorenen Protoplasten als biologische Einheit des Protoplastenbiosensors nachgewiesen werden. Mit Fluazinam, einer fungizid wirkenden Substanz, wurde dennoch ein weiterer Wirkstoff eingesetzt, da dieser in der Landwirtschaft breite Verwendung findet. Zudem ist die fungizide Wirkung auf eine Beeinflussung der Atmung zurückzuführen. Für Fluazinam konnte gezeigt werden, dass dieser Wirkstoff, im Gegensatz zu KCN bzw. SHAM nicht atmungsinhibitierend wirkt, sondern diese entkoppelt. Damit kann der Protoplastenbiosensor nicht nur für den Nachweis atmungshemmender Substanzen verwendet werden, vielmehr kann er auch erste Hinweise auf den Wirkort und die Wirkweise der Substanzen liefern.

Im Rahmen der vorliegenden Arbeit konnte somit ein wesentlicher Beitrag geleistet werden, die biosensorischen Eigenschaften der Protoplasten als biologische Einheit des Protoplastenbiosensors über einen längeren Zeitraum hin zu stabilisieren. Damit kann jetzt die biologische Einheit jederzeit zur Verfügung stehen, was einen deutlichen Fortschritt für die praktische Einsetzbarkeit des Protoplastenbiosensors bedeutet.

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# 7 APPENDIX

## 7.1 Abbreviations

| | |
|---|---|
| ACC | Acclimated |
| EPA | Environmental Protection Agency |
| f. wt. | Fresh weight |
| Glyc 10 | 10% (v/v) glycerol solution |
| Glyc 5 | 5% (v/v) glycerol solution |
| KCN | Potassium cyanide |
| MPP | Mini-protoplasts |
| n | Number |
| NACC | Non-acclimated |
| OECD | Organisation for Economic Co-operation and Development |
| PP | Protoplasts |
| ROS | Reactive oxygen species |
| SD | Standard deviation |
| SHAM | Salicylhydroxamic acid |
| Treh 4 | 0.4M trehalose solution |
| Treh 6 | 0.6M trehalose solution |
| WHO | World Health Organisation |

## 7.2 Equipment

*Autoclave*:
Tuttnauer Systec ELV 3850, Systec GmbH, Wettenberg, Germany

*Centrifuges*:
Beckman Instruments Inc., Palo Alto, CA; Ultracentrifuge L7; SW 60 Ti rotor
Mikroliter, Hettich Zentrifugen, Tuttingen
Minifuge GL, Heraeus Christ GmbH, Osterode

*Flow Cabinet*:
HP 72, Gelaire Flow Laboratories GmbH, Meckenheim, Germany

*Oxygen-Measurement System*:
928 6-Channel Oxygen System, Strathkelvin Instruments Ltd, Glasgow, UK
Interface 928 Oxygen System, Strathkelvin Instruments Ltd, Glasgow, UK
Software: 928 Oxygen System, Strathkelvin Instruments
Magnetic stirrer: Variomag Multipoint HP6, H+P Labortechnik GmbH, Oberschleißheim, Germany
Halogen lamp: Halolux 150, Streppel Glasfaser-Optik, Wermelskirchen, Germany

## 7.3 Chemicals

| Substance | Abbreviation/Formula | Supplier |
|---|---|---|
| Acetone | - | Merck 1.00014 |
| Agar | - | Sigma A-1296 |
| Albumin, Bovine, Fraction V | BSA | AppliChem A1391 |
| Calcium chloride dihydrate | $CaCl_2$ | Merck 1.02382 |
| Cefotaxime sodium | - | Duchefa C0111 |
| Cellulose | - | Merck 1.02324 |
| D(+)-Trehalose dihydrate | - | Sigma T-0167 |
| Dimethyl sulfoxide | DMSO | Sigma D-4540 |
| DL-α-tocopherol phosphoric acid ester disoldium salt | - | Serva 36570 |
| Driselase | - | Sigma D-9515 |
| Fluazinam | - | Zeneca Berkshire, UK |
| Glycerol | - | Roth 3783 |
| 4-(2-Hydroxyethyl)piperazin-1-ethane sulfonic acid | HEPES | AppliChem A1069 |
| Kanamycine | - | Sigma K-4378 |
| L-Ascorbic acid sodium salt | - | Sigma A-7631 |
| Macerozyme | - | Merck 1.2465 |
| Magnesium chloride | $MgCl_2$ | Merck 1.05835 |
| Mannitol | - | Sigma M-9647 |
| 2-(N-Morpholino)ethane sulfonic acid | MES | Biomol 06010 |
| 3-(Morpholino)propanesulfonic acid sodium salt | MOPS | AppliChem A1077 |
| MS-salts | - | Duchefa M0221 |
| Percoll | - | Sigma P-1644 |
| Polyvinylpyrrolidone | PVP25 | Serva 33420 |
| Potassium cyanide | KCN | Aldrich 20,781-0 |
| Potassium hydroxide | KOH | Merck 5033 |
| Salicylhydroxamic acid | SHAM | Aldrich S60-7 |
| Sodium hydrogen carbonate | $NaHCO_3$ | Merck 6329 |
| Sodium sulfite anhydrous | - | Sigma S-0505 |
| Sucrose | - | Merck 1.07651 |

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